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13. ABSTRACT Chronic migraine is a disabling disorder that affects millions of individuals worldwide, and may result from traumatic brain injury. The purpose of this study was to use rodent models of basic migraine mechanisms to characterize new potential treatments for chronic migraine. The scope of the research was to investigate multiple novel potential drug treatments on migraine-related brain excitability, pain-sensing mechanisms, and behavior. The major outcomes of the research were the development of a platform of assays for migraine drug screening, and the identification of new potential therapies. Specifically, we found that the acid sensing ion channel inhibitor amiloride inhibited cortical spreading depression, trigeminovascular activation, and migraine-related hyperalgesia. We also found that delta opioid receptor agonists inhibited spreading depression and migraine related hyperalgesia. Memantine inhibited cortical spreading depression, but did not inhibit trigeminovascular activation or hyperalgesia. Our studies thus identified amiloride and other acid sensing ion channel inhibitors, and delta opioid receptor agonists, as promising candidates for clinical trials as migraine treatments.					
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1. INTRODUCTION:

The subject of this research was the basic mechanisms of chronic migraine and new possible treatments for this common and disabling condition. The purpose of the research was to investigate novel targets for treatment of migraine by studying the responses to pharmacological agents in different rodent models of migraine. The scope of the research was to characterize acid sensing ion channels, glutamate receptors, the sodium-potassium pump, delta opioid receptors, adenosine receptors, and the enzyme casein kinase1 delta as migraine therapeutic targets using cellular, physiological, and behavioral rodent migraine models.

2. KEYWORDS:

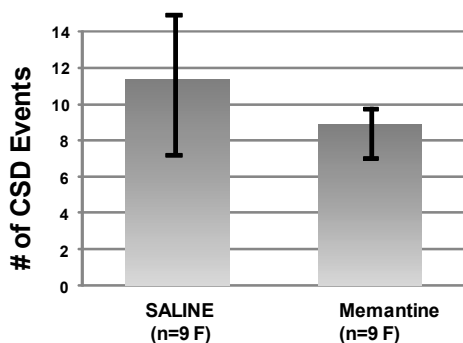
Chronic migraine, NMDA receptors, acid sensing ion channels, sodium-potassium ATPase, , calcium signaling, cortical spreading depression, trigeminovascular, nitroglycerin-evoked hyperalgesia.

OVERALL PROJECT SUMMARY:

Task 1. Characterization of the effects of memantine on migraine models

a. Use optical imaging and electrophysiological techniques to quantify the effects of acute and chronic treatment with memantine on the threshold for induction of CSD, its propagation characteristics, and the accompanying vascular response in C57Bl6 mice (Charles).

Results - These studies were partially completed, but excessive variability in control animals prevented development of clear conclusions. These studies are presently being repeated with new controls, and comparison with other glutamate receptor antagonists.



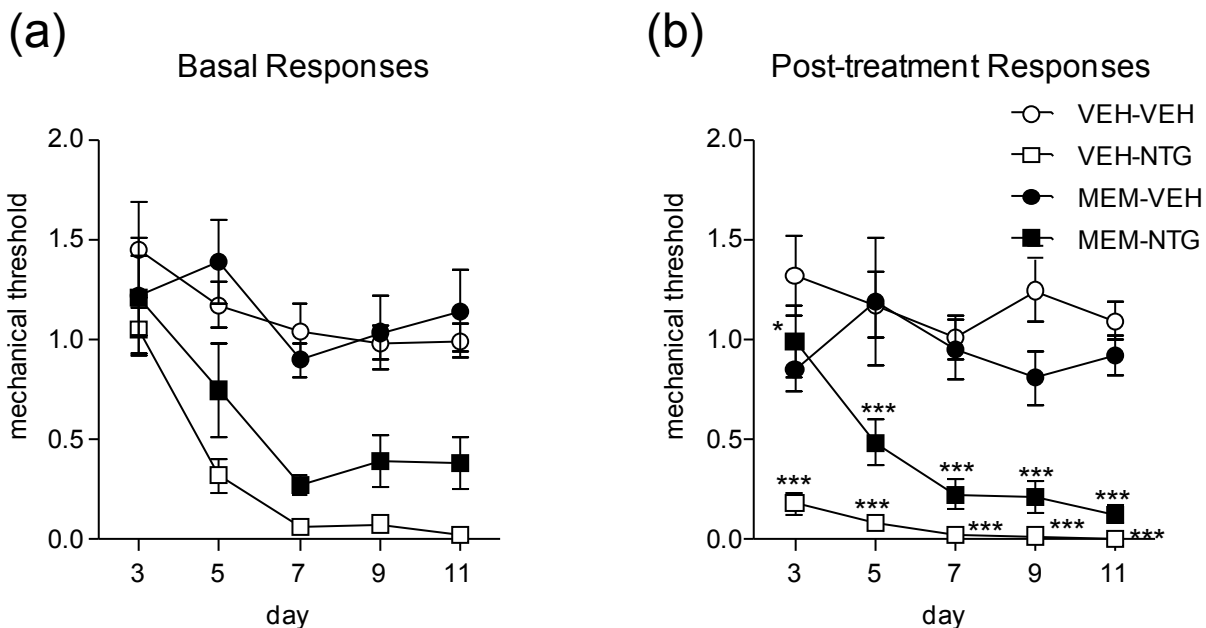
Memantine did not significantly reduce the number of CSD events, but a high level of variability in controls may have confounded this result.

b. Characterize the effects of chronic treatment with memantine on trigeminovascular nociceptive activation in rats (Goadsby).

Results - These studies were completed. It was found that memantine did not inhibit trigeminovascular nociceptive activation in rats when administered intravenously, in contrast to when it was administered iontophoretically directly onto central trigeminal neurons. This result is described in Appendix #1.

c. Determine the effects of chronic treatment with memantine on acute and chronic nitroglycerin-evoked hyperalgesia in mice. (Charles)

Results - These studies were completed. It was found that memantine did not inhibit acute or chronic nitroglycerin-evoked hyperalgesia in mice.



Chronic treatment with memantine did not significantly alter basal or acute NTG-induced hyperalgesia. Male and female C57bl/6J mice were treated with vehicle (VEH) or memantine (MEM 3 mg/kg, ip) daily for 11 days. (a) On days 3, 5, 7, 9, and 11 baseline responses were determined, and mice were then injected with either vehicle (VEH) or NTG (10 mg/kg, ip). 2-way RM ANOVA, $p < 0.01$ drug and time only. (b) Posttreatment responses were assessed 2h following NTG administration. $p < 0.01$ drug, time and interaction. $n = 12/\text{group}$. Long-term, memantine did not block migraine-associated pain in the chronic NTG model.

Task 2. Characterization of the effects of amiloride on migraine models :

a. Use optical imaging and electrophysiological techniques to quantify the effects of acute and chronic treatment with amiloride on the threshold for induction of CSD, its propagation characteristics, and the accompanying vascular response. (Goadsby/Charles)

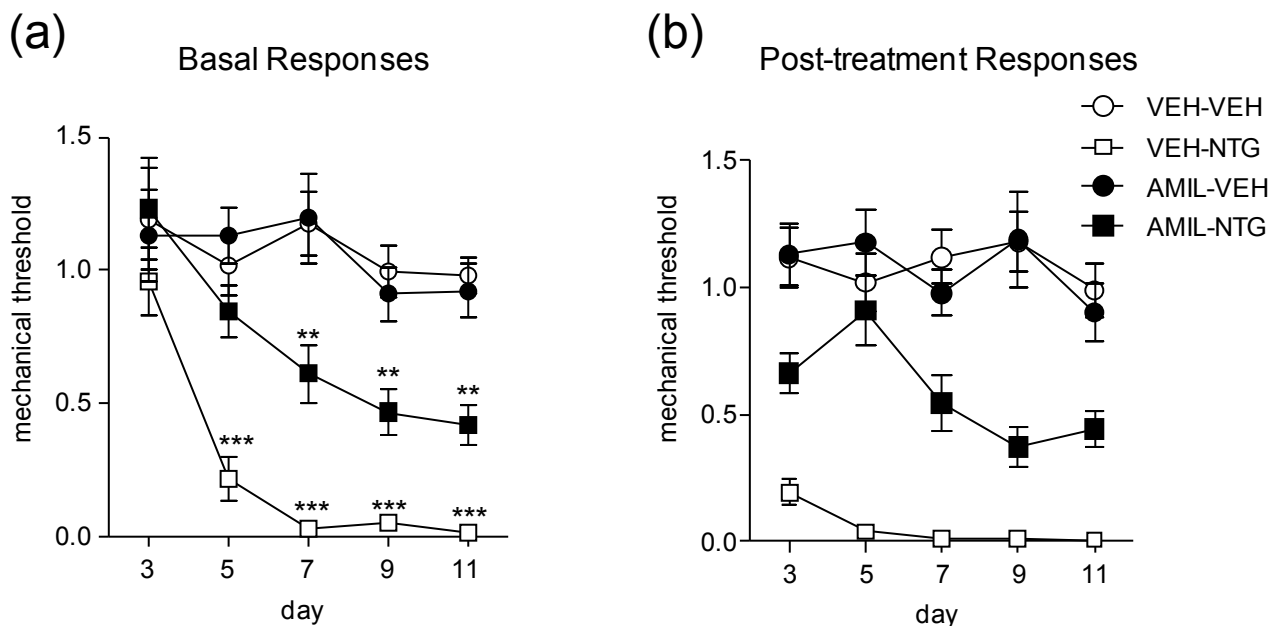
These studies were partially completed. Amiloride was found to block CSD in rats when delivered acutely. Ongoing studies are examining the effects of this and other ASIC blockers on CSD thresholds and propagation characteristics in mice. Appendix # 7, Figure 1A

b. Characterize the effects of acute treatment with amiloride on trigeminovascular electrophysiology and nociceptive mechanisms in rats (Goadsby)

These studies were completed. Amiloride was found to significantly inhibit trigeminovascular activation in rats. Appendix #7, Figure 1G and H

c. Determine the effects of treatment with amiloride on acute and chronic nitroglycerin-evoked hyperalgesia in mice (Charles)

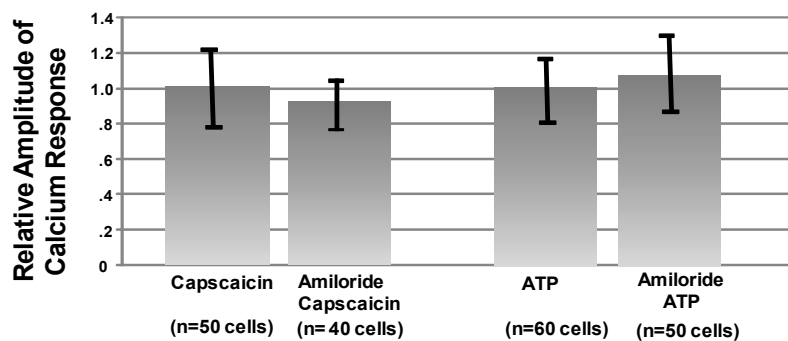
These studies were completed. Amiloride was found to significantly inhibit acute and chronic nitroglycerin-evoked hyperalgesia in mice.



Chronic treatment with amiloride attenuated NTG-induced basal and post-treatment hyperalgesia. Male and female C57bl/6J mice were treated with vehicle (VEH) or amiloride (AMIL 10 mg/kg, ip) daily for 11 days. (a) On days 3, 5, 7, 9, and 11 baseline responses were determined, and mice were then injected with either vehicle (VEH) or NTG (10 mg/kg, ip). 2-way RM ANOVA, $p < 0.01$ drug, time and interaction. (b) Posttreatment responses were assessed 2h following NTG administration. $p < 0.05$ drug and time only. $n = 12/\text{group}$. The development of basal hypersensitivity and acute NTG-evoked hyperalgesia were blocked by the novel migraine prophylactic, amiloride.

d. Characterize the effects of amiloride on the calcium signaling response of trigeminal neurons to nociceptive stimuli (Charles)

These studies were completed. Amiloride was found to have no effect on calcium signaling responses of trigeminal neurons to nociceptive stimuli.



Amiloride did not affect the calcium responses of trigeminal neurons to the nociceptive messengers capsaicin or ATP.

Task 3. Characterization of the effects of canrenone on migraine models

a. Quantify the effects of acute treatment with canrenone on the threshold for induction of CSD, its propagation characteristics, and the accompanying vascular response in mice (Charles)

We were unable to complete these studies, because of substantial difficulties with canrenone solubility resulting in inability to deliver it in consistent doses. This, in combination with opportunities to focus on other attractive targets, led us to focus on other targets. (see below)

b. Characterize the effects of chronic canrenone on trigeminovascular nociceptive activation in mice (Goadsby)

These studies were deferred in light of difficulties in Task 3a. and the negative results of Task 3d.

c. Determine the effects of canrenone on acute and chronic nitroglycerin-evoked hyperalgesia in mice (Charles)

These studies were attempted, but as with Task 3 a, they were not completed because of difficulties with consistent administration of canrenone.

d. Characterize the effects of canrenone on spontaneous and evoked calcium signaling in

astrocytes (Charles) These studies were completed. We found that canrenone had *no significant effect* on spontaneous and evoked calcium signaling in either astrocytes or neurons (Data not shown).

Changes in approach and reasons for change

We were not able to test the effects of the Na⁺/K⁺ ATPase modulator canrenone on rodent migraine models other than cellular signaling in vitro because of difficulties with administration of the compound. We therefore focused our attention on 2 other potential migraine therapeutic targets: delta opioid receptors and adenosine receptors. We are currently pursuing different studies investigating the Na⁺/K⁺ ATPase with different pharmacological and genetic strategies.

4. KEY RESEARCH ACCOMPLISHMENTS

- Development of a platform of rodent assays to investigate basic mechanisms of migraine and to identify and characterize novel potential migraine therapies using these assays (Charles/Goadsby)
- Nitroglycerin-evoked hyperalgesia in mice is a translational model for chronic migraine that can be used to investigate both acute and preventive therapies (Charles)
- The acid sensing ion channel (ASIC) is a potential therapeutic target for migraine (Charles/Goadsby) based on its effects on multiple pre-clinical models of migraine
- Although glutamate receptors are believed to be an important therapeutic target in migraine, memantine, an antagonist of these receptors, did not inhibit trigeminal nociceptive signaling or nitroglycerin-evoked hyperalgesia (Charles/Goadsby)
- The endogenous ouabain antagonist canrenone, a modulator of the Na⁺/K⁺ ATPase, unexpectedly did not have any significant effects on astrocytic or neuronal calcium signaling (Charles)
- Mutations of the gene encoding the enzyme casein kinase 1 delta are associated with familial migraine and advanced sleep phase syndrome and alter migraine-related physiology in rodent models (Charles)
- Delta opioid receptors are an important potential therapeutic target for the acute and preventive treatment of migraine (Charles)
- Caffeine and other modulators of adenosine receptors have significant effects on cortical spreading depression (CSD) and on neurovascular coupling following CSD, identifying these receptors as important potential targets in migraine therapy (Charles)

5. CONCLUSION:

Our studies have established a platform of translational pre-clinical models that will help to identify and characterize new therapies for chronic migraine. Based on these models, we found that acid sensing ion channels are a promising therapeutic target for migraine, and commonly used medications such as amiloride that address this target are candidates for formal clinical trials for migraine. We also used these models to show that the delta opioid receptor, and adenosine receptors are promising targets for migraine therapy. This project also supported work that identified a new migraine gene, casein kinase 1 delta, another potential therapeutic target. The results of these studies can be directly translated into improved care and the development of new therapies for the tens of millions of patients in the U.S. who suffer from this disorder. Our future plans are to move ASIC inhibitors and delta opioid agonists forward into clinical trials, and to further characterize adenosine receptor modulators and casein kinase activators.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Publications

1. Holland PR, Akerman S, Andreou AP, et al. Acid-sensing ion channel 1: a novel therapeutic target for migraine with aura. ***Annals of Neurology*** 72:559-563, 2012.
2. Brennan, K.C., E.A. Bates, R.E. Shapiro, J. Zyuzin, W.C. Hallows, Y. Huang, H.Y. Lee, C.R. Jones, Y.H. Fu, A.C. Charles*, and L.J. Ptacek*. (*Co-corresponding authors) Casein kinase idelta mutations in familial migraine and advanced sleep phase. ***Science Translational Medicine*** , 5: 1-11, 2013.
3. Pradhan, A., Smith, M., McGuire, B., Tarash, I., Evans, C., and A.C. Charles. Characterization of a novel model of chronic migraine. ***Pain***. 155: 269-274, 2014
4. Pradhan, A., Smith, M., Zyuzin, K., and Charles, A. Delta opioid receptor agonists inhibit migraine related pain, aversive state, and cortical spreading depression. ***British Journal of Pharmacology***. 171: 2375-2384, 2014.
5. Charles, A. Migraine: a brain state. *Current Opinion in Neurology*, 26(3):235-9, 2013.
6. Baca, S., Jones, R., Dietz, C., Chen, K., Sofroniew, M., Mody, I, and Charles, A. Cortical spreading depression triggered by selective activation of astrocytes. Submitted, 2015.
7. Kesselman, I., Zyuzin, J., and Charles, A. Adenosine receptors modulate neurovascular uncoupling following cortical spreading depression. In preparation, 2015.

Abstracts

Andreou AP, Sprenger T, Goadsby PJ. Cortical spreading depression-evoked discharges on trigeminothalamic neurons. *Headache*. 2012;52:900.

Monteith TS, Goadsby PJ, Shapiro RE. Eliminating the burden of chronic migraine and posttraumatic headache in military and civilian populations. *Headache*. 2012;52:869.

Hoffmann J, Park JW, Storer RJ, Goadsby PJ. Magnesium and memantine do not inhibit nociceptive neuronal activity in the trigeminocervical complex of the rat. *Journal of Headache and Pain*. 2013;1(Suppl 1):P71.

Modulation of Migraine-Related Cortical Excitability by Adenosine and Its Receptors

I Keselman, J Zyuzin, A Charles Cephalalgia. 33 (S 8), 222-223, 2013

A Screen of Prophylactic Anti-Migraine Medications in a Chronic Nitroglycerin Mouse Model AA

Pradhan, I Tarash, B McGuire, A Charles *Cephalalgia* 33 (S 8), 249-249, 2013.

Invited Presentations

Andrew Charles

June, 2011 - International Headache Society Teaching Course, Berlin, Germany – “Headache mechanisms”

November, 2011 - American Headache Society Scottsdale Meeting, Scottsdale, – “Migraine pathophysiology”

October, 2011 - Mexican Academy of Neurology, Guadalajara, Mexico– “Advances in the basic and clinical science of migraine”

November, 2011 - Turkish Neurology Congress, Antalya, Turkey – “Migraine pathophysiology”

April 2012 - American Pain Society, Honolulu 2012 – “Comparison with migraine and other pain disorders”

March 2012 - American Academy of Orofacial Pain, Pasadena, 2012 – “Headache and orofacial pain”

April 2012 - Japanese Neurological Society, Tokyo, Japan 2012 – “Advances in migraine”

June, 2012 - American Headache Society Annual Scientific Meeting Plenary Lecture, Los Angeles, – “Imaging in Headache”

November, 2012 - American Headache Society Scottsdale Meeting, Scottsdale, -- “Migraine pathophysiology”

June 2013 - International Headache Society Annual Scientific Meeting Presidential Symposium Speaker Boston, “Migraine science brings new opportunities for therapy”

June 2013 - International Headache Society Master School, Tokyo, Japan -- “Basic mechanisms of migraine”

November 2013 - International Headache Academy, Scottsdale, Arizona – “Basic mechanisms of migraine”

April 2014 - American Academy of Neurology Annual Meeting Symposium Speaker, Philadelphia--
“Shifting paradigms in migraine”.

September 2014 - MacDonald Critchley Lecture, European Headache Society Migraine Trust
Meeting, Copenhagen, 2014 --- “What does aura tell us about migraine?”

Peter Goadsby

June 2011 Invited speaker International Headache Congress, Berlin

August 2011 Invited speaker Columbian Neurological Society, Bogota

October 2011 Invited speaker 3rd Asian Regional Headache Congress, Beijing

November 2011 Invited speaker, Coast Research Week, Prince of Wales Hospital, Sydney

March 2012 Speaker Royal Society of Medicine, London

March 2012 Speaker Neurology Grand Rounds, The Alfred Hospital, Melbourne

March 2012 Speaker Neurology Grand Rounds, Westmead Hospital, Sydney

April 2012 Speaker British Association for the Study of Headache, London

June 2012 Speaker Guild of Health Writers, London

August 2012 Organizer Teaching Course, International Association for the Study of Pain, Milan

October 2012 Invited speaker Joint IASP/IHS meeting Hamburg

October 2012 Invited speaker 4th Asian Regional Congress on Headache, Taipei

March 2013 Speaker, International Headache Society Masters School, Tokyo

April 2013 Speaker, Kuwait Neurology Meeting, Kuwait

May 2013 Speaker, Irish Migraine Association, Dublin

June 2013 Speaker, International Neuromodulation Society, Berlin

June 2013 Speaker, International Headache Society, Boston

September 2013, Speaker, World Congress of Neurology, Vienna

November 2013, Speaker, Dutch Headache Society, Delft

January 2014, Speaker International Headache Society Academy, Scottsdale, AZ

March 2014, Australasian Neurology Registrar's Training, Sydney

September 2014, Chair EHMTIC meeting Copenhagen

October 2014, Invited speaker, American Neurological Association

October 2014, Faculty, International Headache Academy Leiden

April 2011 Speaker, American Academy of Neurology Teaching Course

June 2011 Invited Speaker American Headache Society Scientific Meeting, Washington DC

July 2011 Invited speaker Interventional Cardiology Meeting, Boston

November 2011 Invited speaker American Headache Society Teaching Course, Scottsdale

April 2012 Speaker, American Academy of Neurology Teaching Course, New Orleans
June 2012 Invited Speaker American Headache Society Scientific Meeting, Los Angeles
November 2012 Speaker American Headache Society Comprehensive Migraine Course, Houston
November 2012 Invited speaker American Headache Society Teaching Course, Scottsdale
December 2012 Speaker American Headache Society Comprehensive Migraine Course, New York
March 2013 Speaker, American Academy of Neurology Teaching Course
April 2013 Speaker, Mayo Clinical Headache Course, San Francisco
October 2013 Speaker American Headache Society Comprehensive Migraine Course, Minneapolis
November 2013 Invited speaker American Headache Society Teaching Course, Scottsdale
April 2014, IASP/IHS Master School Siena
April 2014 Speaker, American Academy of Neurology Teaching Course
June 2014, BASH/Stoke SPRs training course

7. Inventions, patents and Licenses

None

8. Reportable Outcomes

- These studies have significantly advanced a platform of assays that can be used to identify and characterize chronic migraine therapies. This outcome was reported in the journal ***Pain***.
- Our results provide a new, novel and accessible target for the treatment of migraine- the Acid-Sensing Ion Channels (ASICs), in a context where we have further validated rodent models relevant to migraine. This outcome was reported in the ***Annals of Neurology***.
- We have identified delta opioid receptor agonists as a promising potential migraine treatment. This outcome was reported in the ***British Journal of Pharmacology***.
- We have characterized a new migraine gene -- the gene encoding casein kinase 1 delta. This outcome was published in ***Science Translational Medicine***.

9. Other Achievements

Multiple individuals who worked on this project advanced to achieve new academic positions.

Amynah Pradhan, Ph.D. post-doctoral scholar on this project, obtained a faculty position at the University of Illinois at Chicago School of Medicine

Jekaterina Zyuzin, a technician on this project, was accepted to graduate school at the University of Southern California

Brenna McGuire, a technician on this project, was accepted to medical school at the University of New Mexico

Philip Holland, Ph.D. a post-doctoral scholar has obtained a full-time tenure track position at King's College London in headache-related neuroscience.

10. REFERENCES

None

11. APPENDICES:

1. Hoffmann J, Park JW, Storer RJ, Goadsby PJ. Magnesium and memantine do not inhibit nociceptive neuronal activity in the trigeminocervical complex of the rat. *Journal of Headache and Pain*. 2013;1(Suppl 1):P71.
2. Holland PR, Akerman S, Andreou AP, et al. Acid-sensing ion channel 1: a novel therapeutic target for migraine with aura. *Ann Neurol* 72:559-563, 2012.
3. Brennan, K.C., E.A. Bates, R.E. Shapiro, J. Zyuzin, W.C. Hallows, Y. Huang, H.Y. Lee, C.R. Jones, Y.H. Fu, A.C. Charles*, and L.J. Ptacek*. (*Co-corresponding authors) Casein kinase 1 delta mutations in familial migraine and advanced sleep phase. *Science Translational Medicine* , 5: 1-11, 2013.
4. Charles, A. Migraine: a brain state. *Current Opinion in Neurology*, 26(3):235-9, 2013.
5. Pradhan, A., Smith, M., McGuire, B., Tarash, I., Evans, C., and A.C. Charles. Characterization of a novel model of chronic migraine. *Pain*. 155: 269-274, 2014.
6. Pradhan, A., Smith, M., Zyuzin, K., and Charles, A. Delta opioid receptor agonists inhibit migraine related pain, aversive state, and cortical spreading depression. *British Journal of Pharmacology*. 171: 2375-2384, 2014.

POSTER PRESENTATION

Open Access

Magnesium and memantine do not inhibit nociceptive neuronal activity in the trigeminocervical complex of the rat

J Hoffmann*, JW Park, RJ Storer, PJ Goadsby

From The European Headache and Migraine Trust International Congress
London, UK. 20-23 September 2012

Introduction

Experimental studies with NMDA receptor antagonists, such as magnesium and memantine, have demonstrated the ability of these substances to inhibit nociceptive trigeminal neurotransmission in electrophysiological studies. Despite these promising experimental results clinical trials results have been less than clear. To investigate further this contrast using the open channel blockers, magnesium and memantine, we studied their effects in a model of nociceptive trigeminovascular activation.

Methods

Sprague-Dawley rats were anesthetized with pentobarbital (60 mg/kg-1) and cannulated for physiological monitoring, maintenance of further anesthesia and drug administration. Anesthesia was maintained with intravenous propofol (20-25 mg/kg-1h-1). A cranial window was prepared over the middle meningeal artery (MMA) and a bipolar electrode was placed on the dura mater above the MMA for electrical stimulation. For recording of neuronal activity a tungsten electrode was introduced in the trigeminocervical complex (TCC). Experimental groups received either intravenously administered memantine (10 mg/kg-1), magnesium (100 mg/kg-1) or vehicle.

Results

Magnesium and memantine did not have a significant inhibitory effect on neuronal activity in the TCC. However, blood pressure was significantly reduced after memantine ($31 \pm 5\%$, $p < 0.05$) or magnesium ($49 \pm 11\%$, $p < 0.05$) administration when compared to baseline.

Conclusion

The results indicate that the known inhibitory effect of magnesium and memantine after microiontophoretic application in the TCC could not be reproduced with intravenous administration. This might be a result of the low drug concentration that can be achieved with this route of administration at the relevant site of action. A further increase in dosage is not feasible since the used dosage already led to significant reductions in arterial blood pressure. The results support the clinical observations and provide a possible explanation for the lack of consistent efficacy of these drugs for the treatment of migraine.

Published: 21 February 2013

References

1. Storer RJ, Goadsby PJ: N-Methyl-D-Aspartate receptor channel complex blockers including memantine and magnesium inhibit nociceptive traffic in the trigeminocervical complex of the rat. *Cephalalgia* 2009, **29**(Suppl 1):135.
2. Peikert A, Wilimzig C, Kohne-Volland R: Prophylaxis of migraine with oral magnesium: Results from a prospective, multi-center, placebo-controlled and double-blind randomized study. *Cephalalgia* 1996, **16**(4):257-63.

doi:10.1186/1129-2377-14-S1-P71

Cite this article as: Hoffmann et al: Magnesium and memantine do not inhibit nociceptive neuronal activity in the trigeminocervical complex of the rat. *The Journal of Headache and Pain* 2013 **14**(Suppl 1):P71.

Headache Group - Department of Neurology, University of California San Francisco, USA

Acid-Sensing Ion Channel 1: A Novel Therapeutic Target for Migraine with Aura

Philip R. Holland, PhD,^{1,2} Simon Akerman, PhD,¹ Anna P. Andreou, PhD,¹
Nazia Karsan, MB, ChB,¹ John A. Wemmie, PhD,³ and Peter J. Goadsby, MD, PhD¹

Objective: Migraine with aura is a severe debilitating neurological disorder with few relatively specific therapeutic options.

Methods: We used amiloride, a blocker of epithelial sodium channels, to evaluate its pharmacological potential and explored the biology of a potential mechanism of action in well-established experimental models.

Results: Amiloride was shown to block cortical spreading depression, the experimental correlate of aura, and inhibited trigeminal activation in in vivo migraine models, via an acid-sensing ion channel 1 mechanism. Remarkably, amiloride then demonstrated good clinical efficacy in a small open-labeled pilot study of patients, reducing aura and headache symptoms in 4 of 7 patients with otherwise intractable aura.

Interpretation: The observations here identify an entirely novel treatment strategy for migraine.

ANN NEUROL 2012;72:559–563

Migraine is a common neurological disorder with an annual prevalence of at least 12%,¹ costing nearly \$20 billion in the United States alone.² Approximately 20 to 30% of patients report aura,³ focal neurological disturbances thought to be an expression of the experimental phenomenon of cortical spreading depression (CSD).⁴ The most urgent unmet clinical need in migraine therapeutics is the provision of new approaches to preventive treatment, with only about 1/3 of patients who might require it currently being treated.⁵ Even with knowledge of several mutations in familial forms of migraine,⁶ there is a dearth of novel compounds in phase II development.

Amiloride is known to block the widely expressed epithelial sodium channels (ENaCs)/degenerin gene family,⁷ which includes the acid-sensing ion channels (ASICs). The ASICs represent proton-gated channels that are able to flux Na⁺ and Ca²⁺ and are encoded by 4 genes responsible for 6 different subunits. ASIC1, which is encoded by the *ACCN2*⁷ gene, has been linked to a variety of functions ranging from mechanotransduction to seizure termination⁸ and neuroprotection.⁹ Pharmacological blockade or transgenic deletion of the ASICs has

been shown to protect against tissue damage in models of multiple sclerosis and ischemic brain injury.⁹ Given the known involvement of ASICs in seizurelike behavior,⁸ the induction of tissue hypoxia and disruption by CSD¹⁰ and the further comorbidity data between migraine and epilepsy,¹¹ as well as that a number of anti-epileptic agents are proven preventive treatments in migraine,¹² we proposed that blockade of the amiloride-sensitive ENaCs could be a novel beneficial treatment option for migraine, particularly for patients with aura.¹³

Materials and Methods

Male Sprague Dawley rats and male age-matched wild-type and ASIC 1 knockout (ASIC1^{-/-}) mice on a congenic C57/BL6 background were anesthetized with sodium pentobarbitone and maintained with propofol or isoflurane, respectively. A femoral artery and both femoral veins were cannulated (femoral vein only in mice) for blood pressure monitoring and intravenous infusion. Animals were placed in a stereotaxic frame and physiological parameters were maintained as detailed in the Supplementary Methods.

Cortical steady potentials (direct current [DC]; rat and mouse) and cerebral blood flow (rat only) were recorded (Supplementary Fig 1A) in response to needle prick or K⁺

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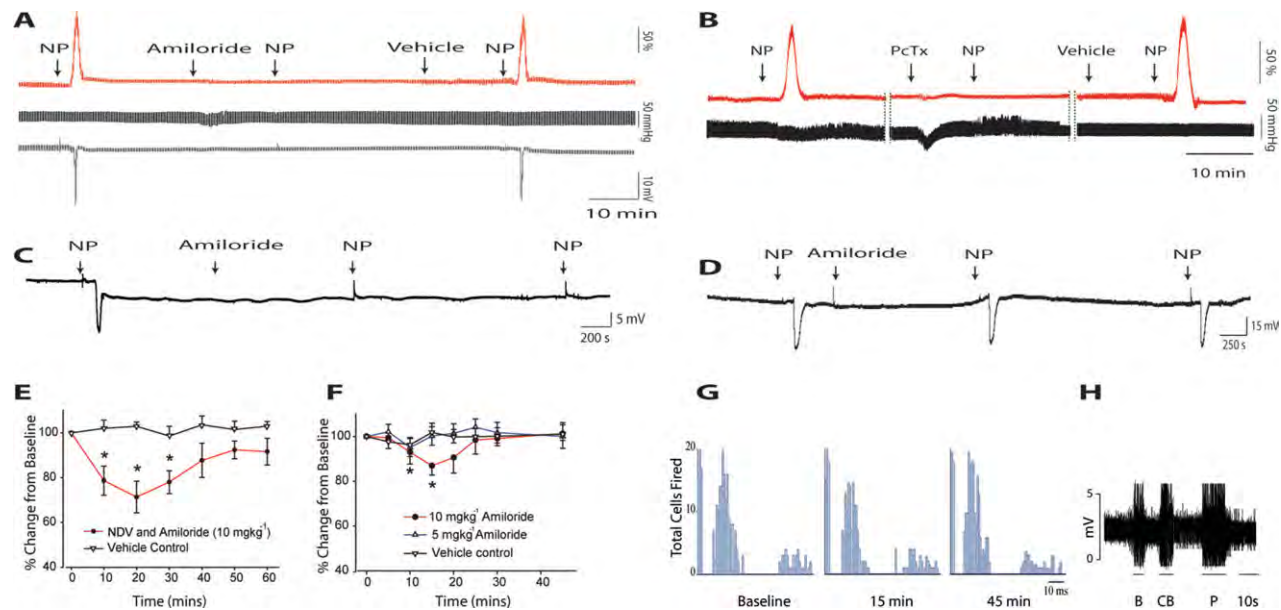


FIGURE 1: Acid sensing ion channel modulation in experimental models of migraine. (A) Regional cerebral blood flow (top), blood pressure (middle), and direct current shift (bottom) recordings from a sample cortical spreading depression (CSD) trace. Amiloride at 10mg/kg^{-1} significantly inhibited needle prick (NP)-induced (6 of 8) CSDs when compared to vehicle controls ($p = 0.007$, Fisher exact test), despite having no significant effect on blood pressure. The lower dose of 5mg/kg^{-1} had no significant effect on CSD propagation ($p = 1.00$, Fisher exact test), inhibiting only 1 of 8. (B) Regional cerebral blood flow (top) and blood pressure recordings from a sample CSD trace. Psalmotoxin (PcTx), the specific acid-sensing ion channel (ASIC) 1a blocker, significantly inhibited NP-induced CSDs (5 of 6) when compared to vehicle control ($p = 0.015$, Fisher exact test), but resulted in adverse blood pressure effects. (C, D) To characterize the likely action of amiloride, transgenic mice with targeted deletion of the ASIC1-producing *ACCN2* gene were compared to age-matched wild-type controls. Amiloride significantly inhibited the number of CSDs propagating in the controls when compared to the *ASIC1*^{-/-} mice ($p = 0.049$, Fisher exact test), with only 1 of 8 blocked in *ASIC1*^{-/-} mice compared to 6 of 9 in controls. (E) Amiloride 10mg/kg^{-1} inhibited neurogenic vasodilation (NVD; $F_{6,42} = 5.1$, $p < 0.001$; $n = 8$) of the middle meningeal artery (MMA), maximally by 29% at 20 minutes postdrug ($t_7 = 4.6$, $p < 0.002$). (F) Effect of amiloride on trigeminal nucleus caudalis neuronal responses to stimulation of the dura mater surrounding the MMA. Amiloride at 10 but not 5mg/kg^{-1} inhibited neuronal responses ($F_{7,49} = 3.15$, $p < 0.01$; $n = 8$), significantly decreasing A-fiber responses at 10 and 15 minutes postdrug when compared to baseline and vehicle controls, maximally by 16%, returning to baseline levels after 20 minutes. (G) Original poststimulus histogram showing decreased A-fiber response 15 minutes post amiloride, returning to baseline levels after 45 minutes. (H) Original example of second-order trigeminal neuronal responses to facial receptive field stimulation, including light brush (B), corneal light brush (CB), and noxious pinch (P). Amiloride had no effect on any aspect of receptive field studied. [Color figure can be viewed in the online issue, which is available at www.annalsofneurology.org.]

application to cortical surface to induce CSD following appropriate rest for refractory periods. Two control CSDs were always initiated to confirm reliable induction followed by a further 2 inductions with either amiloride (10 or 20mg/kg^{-1}) or vehicle randomized to prevent ordering effects. For neurogenic dural vasodilation, a bipolar stimulating electrode was placed on the surface of the cranial window (see Supplementary Fig 1B), and vessel dilation was induced by stimulating at 5 Hz for 10 seconds at increasing currents until maximal response was reached ($100\text{--}200\mu\text{A}$). After 2 baselines, amiloride (10mg/kg^{-1}) was given as a bolus, and the effect on dilation was followed for 1 hour. Extracellular recordings were conducted in the trigeminal nucleus caudalis (TNC) in response to electrical square-wave stimuli (0.6 Hz, 0.1–0.5-millisecond duration, 6–12 V) applied onto the middle meningeal artery (MMA) to activate trigeminal afferents or cutaneous receptive field stimulation following amiloride (5 or 10mg/kg^{-1}) or vehicle. Effects of drug intervention or gene deletion were analyzed using Fisher exact test due to the binary nature of CSD as an

all or nothing event (Prism version 5; GraphPad Software, La Jolla, CA), and total number of CSDs initiated as a result of K^+ induction following amiloride or vehicle control was compared using Student paired t test (SPSS version 12; SPSS Inc, Chicago, IL). The effects of stimulation on vessel diameter were calculated as a percentage increase from the prestimulation baseline. For electrophysiological recordings, the average of 3 baselines for comparisons was used or compared to vehicle control recordings. All intravital and electrophysiological data are expressed as mean \pm standard error of the mean. Statistical analysis was performed using an analysis of variance for repeated measures with Bonferroni post hoc correction for multiple comparisons followed by Student paired t test (SPSS version 12.0) with significance at $p < 0.05$.

Five females and 2 males with medically refractory migraine with prolonged aura were offered amiloride. All patients had medically refractory migraine with persistent aura¹⁴ and experienced discrete worsening of headache and aura symptoms. All patients had standard treatment trials of acetazolamide,

TABLE: Patient Data on the Use of Amiloride

Sex/Age, yr	Aura Phenotype	Length of History, yr	Medications in Addition to Standard Therapy ^a	Dose, mg/day	Outcome	Adverse Events
F/38	Visual, sensory, motor	7	Pizotifen, propranolol, verapamil	10	Nil effect	Mild reversible hyperkalemia
F/35	Visual, sensory, motor, brainstem	6	Pizotifen, propranolol, amitriptyline, verapamil	10	Aura symptoms settled by 75% and headache severity improved by 90%	Nil
F/47	Visual, sensory, motor	5	Pizotifen, propranolol, amitriptyline, methysergide	15	Nil effect	Polyuria
M/39	Visual, sensory, motor	6	Pizotifen, propranolol, amitriptyline, nifedipine	10	Aura symptoms and headache settled by 90%	Nil
F/45	Visual, sensory, motor	3	Pizotifen, propranolol, amitriptyline, nifedipine	10	Aura symptoms settled by 90% and headache severity improved by 80%	Nil
F/44	Visual, sensory, motor	8	Pizotifen, propranolol, amitriptyline	15	Aura symptoms settled by 70% and headache severity by 60%	Nil
M/47	Visual, sensory, motor, brainstem	7	Pizotifen, propranolol, amitriptyline, verapamil	20	Nil effect	Polyuria

^aStandard treatment consisted of acetazolamide, flunarizine, lamotrigine, gabapentin, valproate, and topiramate to maximal doses, which were ineffective or not tolerated, or left significant attacks.
F = female; M = male.

flunarizine, lamotrigine, gabapentin, valproate, and topiramate to maximal doses, which were ineffective or not tolerated, or left significant attacks. All patients had had extensive investigation for underlying secondary causes, particularly prothrombotic states, including brain imaging, without a cause being identified. Patients were followed up from 6 to 24 months for responder outcome.

Results

Cortical needle prick (NP)-induced CSD in rats produced a transient increase in cerebral blood flow in combination with a sharp deflection in DC potential (Fig 1). Administration of amiloride (10mg/kg⁻¹) 15 minutes prior to NP significantly inhibited the number of NP-induced CSDs (6 of 8) when compared to vehicle, but failed to inhibit the number of K⁺-induced CSDs (Supplementary

Results). To characterize the possible mechanism of action of amiloride, the effect of the specific ASIC1a blocker psalmotoxin (PcTx)¹⁵ in the rat model of CSD was studied. PcTx (1:1,000; 1.5ml/kg⁻¹) demonstrated significant efficacy, blocking 5 of 6 NP-induced CSDs. We then explored a novel transgenic mouse that does not express ASIC1 due to deletion of the *ACCN2* gene.¹⁶ Amiloride at 10mg/kg⁻¹ failed to block CSD in 3 control mice, and therefore the higher dose of 20mg/kg⁻¹ was used in mice. Administration of this higher dose significantly inhibited the propagation of NP-induced CSDs in wild-type mice (6 of 9) when compared to ASIC1^{-/-} mice, in which only 1 of 8 was blocked, suggesting that amiloride's anti-CSD actions are likely at least in part due to actions on the ASIC1 subunit and that ASIC1 may play a crucial role in CSD induction.

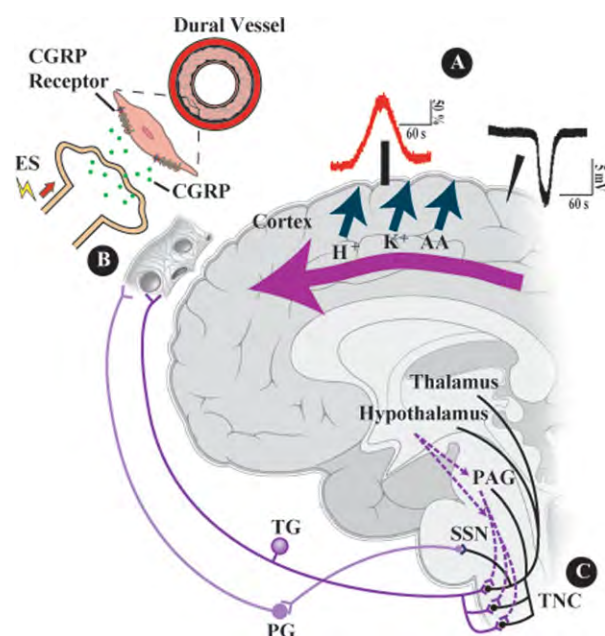


FIGURE 2: The proposed mechanisms of action of amiloride in the trigeminovascular system for the treatment of migraine with or without aura. The pseudounipolar trigeminal nerve conveys information from the dural vessels to the trigeminal nucleus caudalis (TNC) via the trigeminal ganglion (TG). Second-order neurons in the TNC then transmit this information to higher brain structures, including the contralateral thalamus and cortex. Modulatory pathways originating in the cortex, hypothalamus, and periaqueductal gray matter (PAG) among others send descending projections to the TNC, where they can modulate its activity. Amiloride demonstrates a diverse range of actions on the trigeminovascular system. It blocks needle prick-induced cortical spreading depression (CSD; purple arrow), which has been shown to increase *c-fos* in the TNC.¹⁹ The exact mechanism of this activation is unclear, but may be due to direct primary afferent activation or interactions with descending inhibitory pathways (purple dashed lines), resulting in disinhibition.²⁰ (A) CSD results in a dramatic failure of brain ion homeostasis and the release of a variety of agents, including H^+ , K^+ , and arachidonic acid (AA). H^+ and AA are both known to potentiate acid-sensing ion channels,²¹ which may aid the propagation of the spreading depression. In agreement, amiloride is efficacious in the treatment of aura and headache in migraine with aura patients. (B) Electrical stimulation (ES) of the cranial window over the middle meningeal artery (MMA) drives the release of calcitonin gene-related peptide (CGRP) from the prejunctional trigeminal nerve. CGRP activates CGRP receptors on the dural vessels, resulting in dural vasodilation, which is indicative of trigeminal nerve activation. Amiloride inhibits the observed neurogenic dural vasodilation, likely by inhibiting CGRP release, indicating a trigeminal primary afferent action. (C) Amiloride also inhibits TNC neuronal responses to MMA stimulation, demonstrating its ability to further modulate trigeminal nociceptive transmission. The intravenous route of administration may result in amiloride actions in other brain structures that are involved in the pathophysiology of migraine such as the thalamus, hypothalamus, and PAG. PG, pterygopalatine ganglion; SSN, superior salivatory nucleus.

We further studied amiloride's efficacy in 2 established models of trigeminal nociceptive processing in the rat. Electrical stimulation of the thinned cranium produced reproducible dilation of the MMA ($118 \pm 12\%$; $n = 8$) via trigeminally derived local calcitonin gene-related peptide (CGRP) release. This vasodilatation was significantly attenuated by 10mg/kg^{-1} of amiloride, suggesting a direct action on the trigeminal nerve at the level of the dural vasculature (see Fig 1, 2). To test any central actions of amiloride, electrophysiological recordings were measured from 12 populations of neurons in the TNC, all of which were responsive to dural MMA stimulation and had receptive fields within the trigeminal facial dermatome (Supplementary Fig 2). Amiloride at 10 but not 5mg/kg^{-1} significantly inhibited neuronal responses with A-fiber input to dural electrical stimulation when compared to vehicle, but had no effect on spontaneous neuronal firing or receptive field properties, suggesting no tonic action.

The efficacy of amiloride in blocking various animal models of migraine (Fig 2), especially NP-induced CSD, the relative lack of known adverse side effects, and open-label effects of other diuretics suggested its exploratory use in patients suffering from persistent aura who were particularly disabled, and had otherwise medically intractable symptoms. Remarkably, in this difficult to treat group of patients in whom standard treatment options had failed to provide sufficient symptomatic control, and whose outcome we audited for this report, amiloride substantially reduced both frequency of aura and headache severity in 4 of 7 patients receiving 10 to 20mg/day^{-1} , followed up for between 6 and 24 months (Table).

Discussion

Taken together, the data offer a strong indication that the ASIC1 subunit may offer a therapeutic target in migraine with aura, if not migraine more generally. The data support the development of specific compounds to target this receptor and the conducting of randomized placebo-controlled trials. Interestingly, amiloride was able to block NP-induced CSDs and showed further efficacy in 2 diverse models of trigeminovascular activation, suggesting multiple relevant sites of action. In agreement, amiloride reduced both the frequency of aura and the severity of pain in migraine with aura patients, suggesting a possible beneficial effect in migraine without aura. The failure of amiloride to inhibit K^+ -induced CSDs despite its efficacy in treating migraine aura suggests an interesting differentiation between the 2 experimental CSD-induction models, as previously highlighted.¹⁷ Recent

data have indicated 2 distinct mechanisms for stimulating CGRP release from trigeminal ganglia neurons, either via a triptan responsive Ca^{2+} -dependent mechanism, or a likely Na^{+} -dependent proton-mediated activation of ASICs that is triptan nonresponsive.¹⁸ Although the exact mechanisms involved need to be dissected, the clear efficacy of amiloride in experimental models and the indication of a clinical effect in migraine with aura patients suggest that ASICs are an option for a major therapeutic advance in a common and highly disabling neurological disorder.

Authorship

P.R.H. and S.A. contributed equally to this study.

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Potential Conflicts of Interest

P.R.H.: speaking fees, Almirall, Headache Cooperative of the Pacific. S.A.: consultancy, MSD, MAP Pharma/Allergan; employment, Eli Lilly; speaking fees, MSD; travel expenses, International Headache Society, American Headache Society. P.J.G.: board membership, Allergan, Colucid, MAP, Merck Sharpe & Dohme, eNeura, Neuraxon, Autonomic Technologies, Boston Scientific, Eli Lilly, Medtronic, Linde, Bristol-Myers Squibb; consultancy, Pfizer, UCSF; grants/grants pending, GlaxoSmithKline, MAP, MSD, eNeura, Amgen; speaking fees, MSD, Pfizer, Allergan, Mennarini; paid educational presentations, American Headache Society.

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MIGRAINE

Casein Kinase I δ Mutations in Familial Migraine and Advanced Sleep Phase

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Migraine is a common disabling disorder with a significant genetic component, characterized by severe headache and often accompanied by nausea, vomiting, and light sensitivity. We identified two families, each with a distinct missense mutation in the gene encoding casein kinase I δ (CKI δ), in which the mutation cosegregated with both the presence of migraine and advanced sleep phase. The resulting alterations (T44A and H46R) occurred in the conserved catalytic domain of CKI δ , where they caused reduced enzyme activity. Mice engineered to carry the CKI δ -T44A allele were more sensitive to pain after treatment with the migraine trigger nitroglycerin. CKI δ -T44A mice also exhibited a reduced threshold for cortical spreading depression (believed to be the physiological analog of migraine aura) and greater arterial dilation during cortical spreading depression. Astrocytes from CKI δ -T44A mice showed increased spontaneous and evoked calcium signaling. These genetic, cellular, physiological, and behavioral analyses suggest that decreases in CKI δ activity can contribute to the pathogenesis of migraine.

INTRODUCTION

Migraine is a complex neurological disorder that affects 20 to 30% of the population (1, 2). About one-third of patients with migraine experience aura: a visual, sensory, or language disturbance preceding the migraine headache (3). Family and epidemiological studies provide strong evidence for a genetic contribution to migraine, although environmental factors also play a role (4, 5). Mutations in three different genes have been identified to cause familial hemiplegic migraine, an uncommon variant of migraine associated with unilateral weakness (6–8). The genetic basis for common forms of migraine (with and without aura) is less clear. Multiple susceptibility loci for migraine have been identified (9–12), and a potassium channel gene has been linked to migraine with aura in a single family (13). These studies suggest that a variety of genetic alterations affecting various aspects of brain excitability can lead to similar migraine phenotypes.

Here, we describe a family that presented for clinical evaluation of familial migraine with aura. In addition to migraine, the family members exhibited circadian patterns consistent with familial advanced sleep phase syndrome (FASPS), in which individuals go to sleep unusually early in the evening and wake early in the morning. We identified a mutation (threonine to alanine, T44A) in casein kinase I δ (CKI δ) as a cause of FASPS in this family (14). CKI δ is a ubiquitous serine-threonine kinase that phosphorylates the circadian clock protein Per2 and many other proteins involved in brain signaling (15). In vitro studies showed that the T44A alteration in CKI δ resulted in reduced enzyme function in vitro, and both mice and flies expressing the mutant allele had significant sleep-wake cycle alterations (14). Given that migraine with aura was a prominent component of the presenting phenotype

of the family in whom the CKI δ mutation was identified, we investigated a potential role for CKI δ in migraine.

RESULTS

Human phenotypes

Fourteen members of the initial family with CKI δ allele-associated FASPS (K5231) were available for structured interview, physical examination, and CKI δ genotyping, including four spouses of affected individuals with potentially affected offspring (Fig. 1A). The proband (#33376) carried the CKI δ -T44A allele and met diagnostic criteria both for FASPS and for migraine with aura (MA) by ICHD-2 (International Classification of Headache Disorders, Second Edition) criteria (16). Of the 13 other family members that we characterized, 5 individuals carried the CKI δ -T44A allele, and all of these also met diagnostic criteria for either MA or migraine without aura (MO). Three individuals who were offspring of CKI δ -T44A allele carriers, but did not carry the allele themselves, also met diagnostic criteria for MA (Fig. 1A).

The clinical characteristics of migraine for the CKI δ -T44A allele carriers were unremarkable with respect to age of onset, time of day of attack onset, duration and character of aura symptoms, presence of cutaneous allodynia (the experience of innocuous touch as uncomfortable or tender), and duration of headache per attack. The proband (#33376) developed chronic migraine (more than 15 headache days per month) from her early 20s through her early 30s and then again between her early 40s and her early 50s with spontaneous remission each time. Brain magnetic resonance imaging studies for three individuals (#33373, #33374, and #33376) were unremarkable apart from evidence of a previous pituitary tumor excision in #33373. The proband's mother (#33374) had French-Canadian ancestry.

After identifying CKI δ -T44A in K5231, we sequenced the gene encoding CKI δ in blood samples from more than 70 FASPS probands and identified a second mutation in a single individual (#35315 in kindred 5579) that predicts a histidine-to-arginine change at position 46 (H46R) (Fig. 1B and fig. S1), two amino acids downstream from

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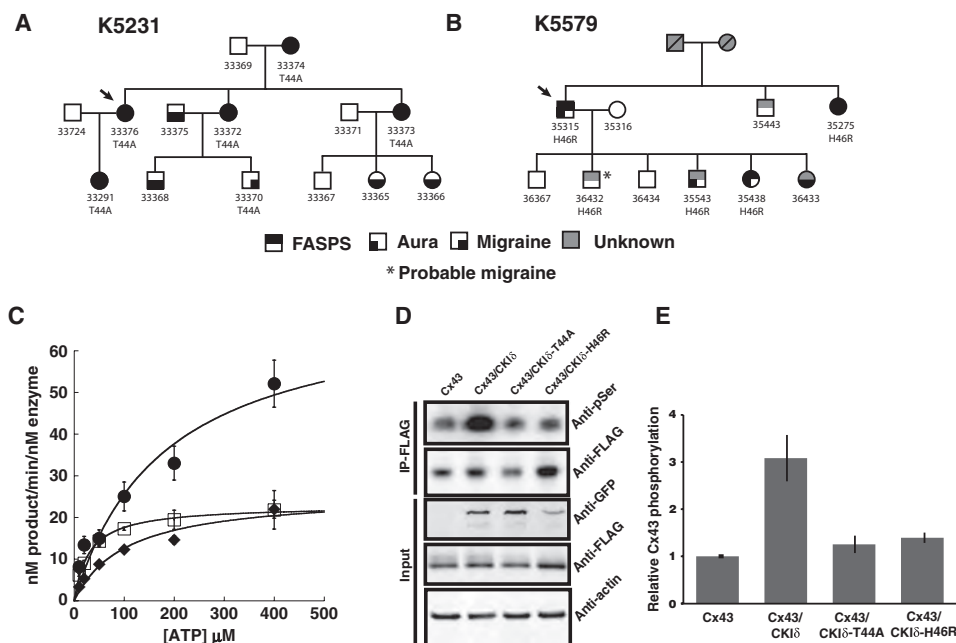


Fig. 1. Migraine kindreds with mutations in the gene encoding CKI δ and in vitro enzymatic analysis. (A) Kindred 5231 segregating the T44A CKI δ allele. (B) Kindred 5579 segregating the H46R CKI δ allele. Phenotypes are as noted, and probands are indicated by arrows. "Migraine" indicates symptoms consistent with ICHD-2 migraine without aura. "Aura" indicates symptoms consistent with ICHD-2 migraine aura. (C) Saturation kinetic analysis of wild-type (●), T44A (□), and H46R (◆) CKI δ 317 using α -casein, PER2 peptide, and ATP. (D and E) Phosphorylation assays. Transiently transfected connexin43 is phosphorylated when coexpressed with CKI δ wild type. FLAG-connexin43 was expressed alone or with CKI δ wild type, T44A, and H46R in HEK293 cells. FLAG-connexin43 was immunoprecipitated by anti-FLAG conjugated to agarose at 4°C for 4 hours. These extracts were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and phosphorylated and dephosphorylated connexin43 was detected by Western blotting with anti-phosphoserine and anti-FLAG antibodies, respectively. Input blot proteins were detected with anti-FLAG, anti-GFP (green fluorescent protein), and β -actin antibody. Relative phosphorylation was normalized to connexin43 protein levels, quantified, and analyzed by LI-COR image software. Data are means \pm SEM ($n \geq 4$). * $P < 0.05$, analysis of variance (ANOVA) followed by Tukey test.

the T44A substitution. This family is primarily of English and Irish descent. Information regarding the migraine phenotype in the family with the H46R mutation was obtained by an e-mail survey and by follow-up e-mail or phone contact. Phenotypic data were analyzed blind to genotypes and FASPS phenotypes. Four of the five family members who carried the H46R allele had MO, MA, or migraine aura without headache, and the fifth had probable migraine by ICHD-2 criteria. Of five family members who did not carry the H46R allele, one had MA. We examined the 1000 Genomes database, National Heart, Lung, and Blood Institute (NHLBI) ESP6500 variant data set, and 69 public genomes from Complete Genomics for the T44A and H46R alleles that we had identified in the migraine families and found neither allele in these controls. No other coding variants in the first 200 amino acids of CKI δ were found in Caucasian samples available in public databases. We also sequenced DNA from an additional 250 Northern European controls from our laboratories and found these alleles in none of them. Furthermore, the protein sequences of CKI δ are extremely conserved across species from human to fruit fly to single-cell algae, and T44 and H46 are absolutely conserved (fig. S1). Thus, these two CKI δ alleles were not present in more than 2600 control chromosomes. We estimate that the chances of coincidentally having mutations in the same

ultraconserved region in two families with the same phenotype are extremely small.

We previously showed that the CKI δ -T44A protein has reduced kinase activity in comparison to the wild-type enzyme (14). We repeated these experiments for the CKI δ -H46R isoform (Fig. 1C). CKI δ -H46R also showed decreased kinase activity with a V_{\max} of (mean \pm SEM) $104.64 \pm 6.61 \text{ nM min}^{-1} \text{ nM}^{-1}$ compared to $198.27 \pm 11.23 \text{ nM min}^{-1} \text{ nM}^{-1}$ for the wild-type enzyme, representing a 53% reduction in activity ($P < 0.01$, $n = 3$). The Michaelis constant (K_m) was $43.38 \pm 6.17 \text{ }\mu\text{M}$ for CKI δ -H46R and $36.48 \pm 7.79 \text{ }\mu\text{M}$ for wild-type CKI δ (Table 1). This decrease in V_{\max} suggests that the mutation causes a reduction in the catalytic rate of phosphotransfer. There may be a subtle effect on enzyme recognition of the substrate as the K_m of the mutant kinase activity is decreased with PER2 peptide but is not significantly different with casein or adenosine triphosphate (ATP). We also tested the ability of wild-type and mutant forms of CKI δ to phosphorylate connexin43, a known substrate hypothesized to be relevant to migraine (17). Both the T44A and H46R mutant forms of CKI δ phosphorylated connexin43 to a lesser degree than did wild-type CKI δ (Fig. 1, D and E).

Increased nitroglycerin-induced hyperalgesia in CKI δ -T44A mice

To further examine whether mutant CKI δ can cause migraine symptoms, we tested CKI δ -T44A transgenic mice for their susceptibility to a migraine-like phenotype.

Nitroglycerin (NTG) evokes peripheral mechanical and thermal hyperalgesia in mice and humans (18, 19). We quantified NTG-evoked mechanical and thermal hyperalgesia in CKI δ -T44A mice and wild-type littermate controls. Mechanical and thermal nociception thresholds were determined with von Frey filaments and the Hargreaves radiant heat assay, before and for a 4-hour period after intraperitoneal injection of NTG (1, 3, 5, 7, or 10 mg/kg). During the 4-hour follow-up, the mechanical thresholds were significantly lower in CKI δ -T44A mice than in wild-type controls ($n = 14$ wild-type and 15 CKI δ -T44A, $P < 0.01$ for each dose with a linear mixed-effects model; Fig. 2 and table S1). After NTG injection, CKI δ -T44A mice were also more sensitive to thermal stimuli than wild-type siblings, with a significantly slower recovery from thermal hyperalgesia ($n = 24$ CKI δ -T44A and $n = 21$ wild-type mice; $P < 0.01$ for each dose with a linear mixed-effects model; Fig. 2 and table S2). Because migraine is more common in women than men, we compared female and male CKI δ -T44A mice with wild-type animals 90 min after NTG (5 mg/kg) injection. At this dose and time point, female, but not male, CKI δ -T44A mice showed a significant reduction in latency to paw withdrawal compared to wild-type animals (males: wild-type, $0.99 \pm 0.69 \text{ s}$; CKI δ -T44A, $2.46 \pm 0.7 \text{ s}$; $P = 0.38$; females: wild-type, $0.69 \pm 0.75 \text{ s}$; CKI δ -T44A, $2.87 \pm 0.58 \text{ s}$; $P = 0.008$,

Table 1. Biochemical characterization of the migraine-associated CKIδ mutant enzymes. *n* ≥ 3 independent experiments with three replicates each; all measurements shown are ±SEM.

Substrate	α-Casein		PER2 peptide		ATP	
	<i>V</i> _{max} (nM min ^{−1} nM ^{−1})	<i>K</i> _m (μM)	<i>V</i> _{max} (nM min ^{−1} nM ^{−1})	<i>K</i> _m (μM)	<i>V</i> _{max} (nM min ^{−1} nM ^{−1})	<i>K</i> _m (μM)
Wild-type CKIδΔ317	198.3 ± 11.2	36.5 ± 6.2	184.0 ± 24.4	635.8 ± 131.4	71.7 ± 14.7	180.6 ± 80.1
CKIδ-T44AΔ317	130.3 ± 6.7*	29.4 ± 4.8	80.1 ± 12.6*	230.6 ± 76.5*	22.9 ± 0.4*	30.0 ± 2.2
CKIδ-H46RΔ317	104.6 ± 6.6*	43.4 ± 7.8	49.5 ± 8.8*	371 ± 120.4*	25.8 ± 3.2*	104.7 ± 33.3

**P* < 0.05 (Student's *t* test).

ANOVA with post hoc Tukey test), suggesting that both sex and genotype may contribute to the difference in thermal hyperalgesia. Rotarod testing showed no difference in initial or repeat performance for CKIδ-T44A mice and wild-type mice (fig. S2), suggesting that differences in response to thermal and mechanical stimulation were not due to differences in motor function or learning.

Neurons in the trigeminal nucleus caudalis (TNC) and laminae I to V of upper cervical dorsal horn (C1 and C2) receive craniofacial pain input and are believed to be activated during migraine (20–23). We administered NTG (5 mg/kg) to CKIδ-T44A (*n* = 7) and wild-type littermates (*n* = 6) and measured the number of activated neurons in TNC, C1, and C2 with Fos immunohistochemistry. After NTG treatment, we found a significant increase in the number of Fos-positive nuclei in all three areas in CKIδ-T44A animals compared to wild-type littermates in TNC (mean ± SEM, 34 ± 2 versus 11 ± 1), C1 dorsal horn (33 ± 3 versus 17 ± 2), and C2 dorsal horn (28 ± 2 versus 14 ± 2) (Fig. 3). The number of Fos-positive nuclei was not significantly different in laminae VI to X.

Reduced threshold for induction of cortical spreading depression in CKIδ-T44A mice

To investigate the effects of CKIδ mutations on migraine-related cortical excitability, we used optical imaging and electrophysiological recording to measure cortical spreading depression (CSD) in vivo in CKIδ-T44A mice (Fig. 4, A and B). Potassium chloride (KCl) was applied to the cortex at increasing volumes until CSD was induced to determine the threshold for evoking CSD (24). CSD thresholds were significantly lower in CKIδ-T44A compared to wild-type mice (Fig. 4D). CSD velocities were similar in both groups (Fig. 4C). After we determined these threshold values, repeat inductions were performed at threshold stimulus every 10 min

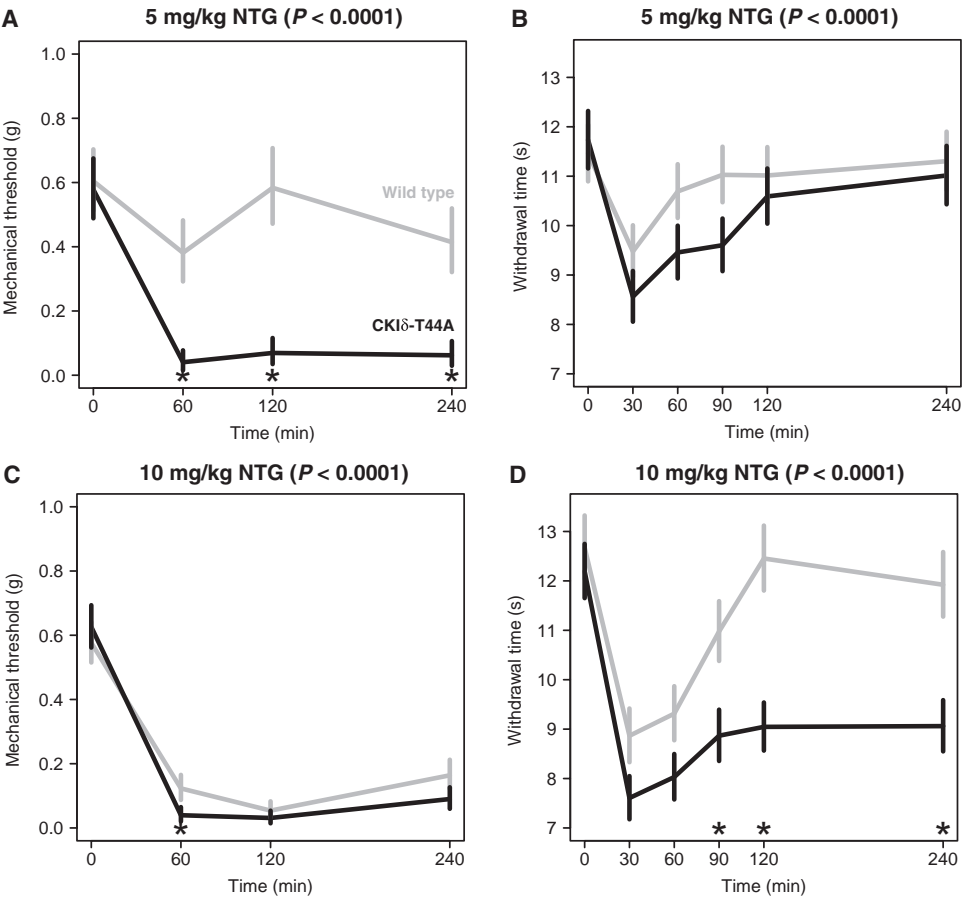


Fig. 2. Differential effects of mechanical and thermal stimuli in response to NTG for wild-type and CKIδ-T44A mice. Data shown (means ± SEM) were compared with a linear mixed-effects model. Gray lines, wild type; black lines, CKIδ-T44A. Differences across time between CKIδ-T44A and wild type are significant at each NTG dosage (*P* values displayed for each panel). Asterisks indicate time points with significant difference between genotypes. (A and C) Mechanical hyperalgesia measured with von Frey hair (Semmes-Weinstein monofilament). (B and D) Thermal hyperalgesia in CKIδ-T44A and wild-type mice in response to NTG [5 mg/kg (B) or 10 mg/kg (D)] intraperitoneal injection, measured with radiant heat assay (Hargreaves test). **P* < 0.05 (see tables S1 and S2 for detailed data).

for the next 60 min. Despite the difference in the thresholds, there was no significant difference in the number of successful CSD inductions between the two groups (Fig. 4), showing that the lower CSD thresholds in CKIδ-T44A mice were reproducible over time. The number of CSDs induced in response to a constant stimulus is also used to

measure CSD susceptibility (25). During 60 min of continuous application of KCl, CKI δ -T44A mice showed a significantly greater number of CSD events than did wild-type mice (Fig. 4E).

Enhanced CSD-associated arterial dilation in CKI δ -T44A mice

Meningeal and cortical surface arteries react to CSD (26, 27). Activation of perivascular nociceptive afferents has been proposed as a mechanism through which CSD activates the TNC (21, 23). Pre-CSD baseline arterial diameter was the same in CKI δ -T44A and wild-type mice (Fig. 5). In both CKI δ -T44A and wild-type mice, CSD was associated with multiphasic changes in arterial diameter: an initial dilation coincident with or ahead of the CSD wavefront, a constriction corresponding to the tissue depolarization, and a second dilation before a return toward baseline (27) (Fig. 5). Cortical surface arteries were more dilated in all phases of CSD in CKI δ -T44A mice compared with wild-type mice (Fig. 5C). We found no correlation between the magnitude of CSD-associated changes in vascular caliber and CSD threshold (177 diameter measurements in 15 mice where such measurement was possible: dilation 1: $r = -0.009$, $t = 0.11$, $t_{\text{crit}} = 1.97$, $P = 0.9$; constriction: $r = 0.11$, $P = 0.13$; dilation 2: $r = 0.02$, $P = 0.69$; post-CSD baseline: $r = -0.006$, $P = 0.92$), suggesting that these vascular differences were not due to the lower CSD thresholds in CKI δ -T44A mice.

Amplitude, duration, and velocity of CSD. We compared the amplitude and duration of both intrinsic signal and electrophysiological variables associated with CSD in CKI δ -T44A and wild-type mice (table S3). There was no significant difference in intrinsic signal or dc (direct current) shift amplitude. Although the durations of intrinsic signal changes and dc shifts were significantly shorter in CKI δ -T44A mice, there was a significant correlation between duration of dc shift and CSD threshold in all mice ($r = 0.6$, $t = 3.83$, $t_{\text{crit}} = 2.05$, $P = 0.0007$, $n = 25$ mice) and in CKI δ -T44A and wild-type mice considered separately (CKI δ -T44A: $r = 0.56$, $t = 2.68$, $t_{\text{crit}} = 2.13$, $P = 0.02$, $n = 17$ mice; wild type: $r = 0.65$, $t = 2.56$, $t_{\text{crit}} = 1.83$, $P = 0.03$) (fig. S4). We ascribed this difference in duration to the significant difference in thresholds between the two groups. The velocity of CSD was similar between CKI δ -T44A and wild-type mice, with no significant difference detected (Fig. 4C).

Sex differences in CSD susceptibility. We previously identified a reduced threshold for CSD in female compared to male C57Bl/6 mice (24), and others have found a reduced CSD threshold in female mice expressing familial hemiplegic migraine mutations (28). We observed a gradient of CSD thresholds by sex and genotype, with the lowest thresholds in female CKI δ -T44A and the highest in male wild-type animals (fig. S3). ANOVA analysis of CSD thresholds by gender and genotype revealed a significant difference over the four combinations (female CKI δ -T44A, male CKI δ -T44A, female wild-type, and male wild-type; one-way ANOVA, $F = 3.74$, $F_c = 2.70$, $P = 0.01$). Post hoc Tukey test showed that the only significantly different paired comparison was between female CKI δ -T44A and male wild-type mice. We concluded that, although there may be a trend toward sex differences in our data set, the main driver of differences was genotype.

Calcium signaling in astrocytes from CKI δ -T44A mice

Astrocyte Ca^{2+} signaling occurs in conjunction with CSD and may mediate some of the vascular changes associated with CSD (29). The primary connexin expressed by astrocytes, connexin43, is a mediator of astrocyte signaling and can be modulated by CKI δ (30). We found that a greater number of cultured astrocytes from CKI δ -T44A mice

showed spontaneous Ca^{2+} oscillations and intercellular Ca^{2+} waves than did astrocytes from wild-type mice (Fig. 6). We also investigated the effects of exposure to medium without extracellular Ca^{2+} (0 Ca^{2+}), a condition that evokes intercellular waves of increased intracellular Ca^{2+} in astrocytes by causing release of ATP (31, 32). Astrocytes from CKI δ -T44A mice showed an increased Ca^{2+} response to 0 Ca^{2+} medium compared to wild-type astrocytes, with a greater number of sites of initiation of intercellular Ca^{2+} waves and a greater extent of propagation of intercellular Ca^{2+} waves (Fig. 6, B and C). We did not, however, detect a significant increase in ATP release to this stimulus in astrocytes from CKI δ -T44A mice (fig. S5).

DISCUSSION

We identified two distinct missense mutations in the gene encoding CKI δ that cosegregated with migraine in two independent families with

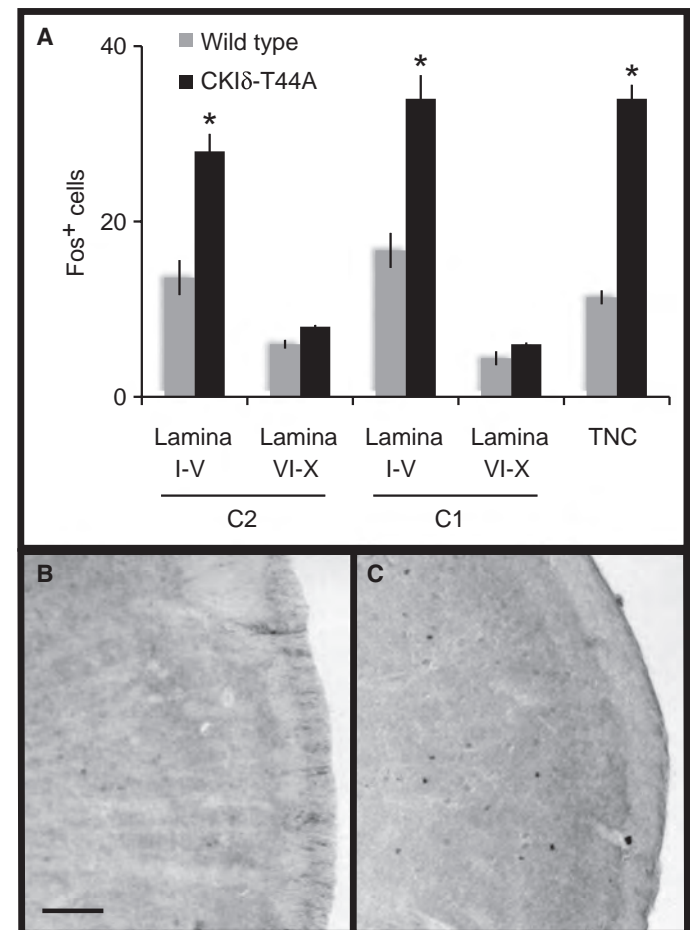


Fig. 3. NTG-induced Fos expression in the spinal cord and TNC. (A) Quantification of Fos-immunoreactive nuclei in the cervical spinal cord lamina I to V, VI to X, and TNC in age-matched wild-type ($n = 6$) and CKI δ -T44A transgenic ($n = 7$) mice 2 hours after treatment with systemic NTG (5 mg/kg). Data are expressed as average numbers of Fos⁺ cells per section. * $P < 1 \times 10^{-5}$, two-way Student's t test. (B and C) Representative sections showing Fos immunoreactivity in wild-type (B) and CKI δ -T44A (C) TNC.

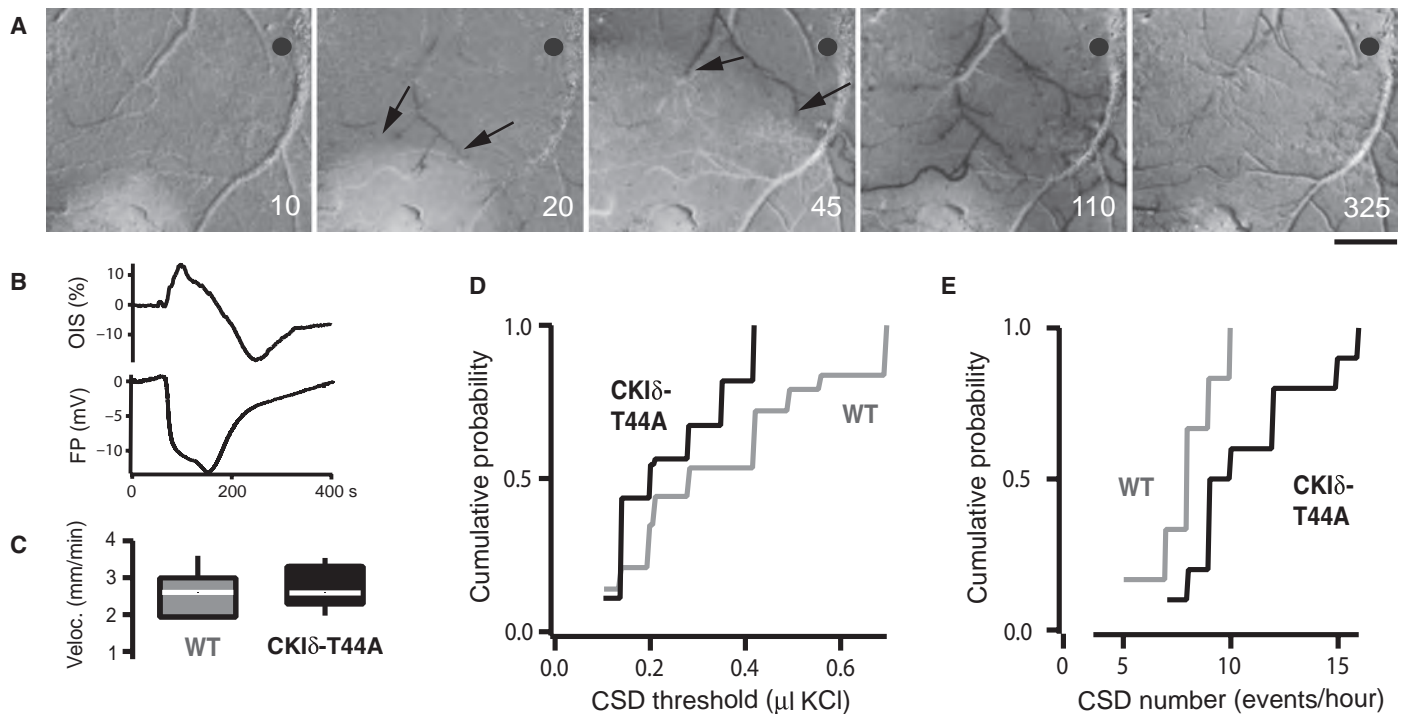


Fig. 4. Increased susceptibility to CSD in CKI δ -T44A mice. (A) Normalized reflectance images ($r/r_0 \times 100$) show CSD-associated optical intrinsic signal (OIS) changes in a CKI δ -T44A mouse brain. KCl-induced CSD causes changes in reflectance that propagate concentrically outward from the source at about 3 mm/min. Reduced reflectance precedes brightening, followed by profound darkening (image 4) before return toward baseline. Reduced arterial reflectance shows the arterial dilation (arrows) that precedes CSD. Numbers indicate the seconds after CSD induction. Scale bar, 500 μ m. Gray circle, field potential electrode and optical measurement location. (B) OIS and field potential traces from the same experiment as in (A), showing OIS changes and the direct current field potential shift of CSD. OIS trace shows percentage change from pre-CSD. (C) CSD velocity in CKI δ -T44A mice and wild-type (WT) littermates. Median [interquartile range (IQR)] velocity was 2.7 mm/min (2.0 to 3.0 mm/min) in wild-type littermates

and 2.6 mm/min (2.3 to 3.2 mm/min) in CKI δ -T44A mice ($n = 6$ wild-type mice, 17 CSD; 9 CKI δ -T44A mice, 29 CSD; $P = 0.77$, Mann-Whitney-Wilcoxon test). (D) Threshold for CSD in wild-type and CKI δ -T44A mice. Median (IQR) threshold was 0.28 μ l (0.2 to 0.49 μ l) of 1 M KCl in wild-type littermates compared to 0.20 μ l (0.14 to 0.35 μ l) for CKI δ -T44A mice ($n = 11$ wild-type mice, 43 CSDs; 14 CKI δ -T44A mice, 55 CSDs; $P = 0.001$, Mann-Whitney-Wilcoxon test). There was no significant difference in the ratio of successful CSD inductions in wild-type (3.90:6) versus CKI δ -T44A (3.92:6) mice ($n = 11$ wild-type, 14 CKI δ -T44A mice; $P = 0.34$, Mann-Whitney-Wilcoxon test). (E) Elicitation of CSD in response to constant stimulation over an hour in CKI δ -T44A mice and their wild-type littermates. Wild-type mice had median (IQR) of 8.0 CSD/hour (6.5 to 9.25 CSD/hour) and CKI δ -T44A mice had 9.5 CSD/hour (8.75 to 12.75 CSD/hour) ($n = 6$ wild-type mice, 10 CKI δ -T44A mice; $P = 0.03$, Mann-Whitney-Wilcoxon test).

advanced sleep phase syndrome. The mutations (T44A and H46R) occur in a conserved catalytic domain and reduce CKI δ kinase activity compared to wild-type. Mice expressing the CKI δ -T44A allele are more sensitive to pain and have increased activation of neurons in the TNC after treatment with a common migraine trigger, NTG. CKI δ -T44A mice also show a reduced threshold for CSD, increased frequency of CSD episodes, and greater arterial dilation with CSD. Astrocytes from CKI δ -T44A mice show increased spontaneous and evoked calcium signaling.

Migraine is frequent (~12% prevalence) in the general population, and it is unclear at this point what underlying genetic variants contribute to this frequency. Multiple loci that increase susceptibility to migraine have been identified (9–11, 13), and environmental factors could also play a role. In some cases, migraine is almost certainly caused by common complex genetic risk alleles segregating in the population. It is also possible that some alleles are autosomal dominant or autosomal recessive, but can be difficult to recognize as such because of polygenic genetic risk alleles segregating in the same families harbor-

ing the Mendelian alleles. This may be the case for the kindred reported here in which there are individuals with migraine who did not carry the migraine-associated CKI δ allele.

Of 11 mutation carriers, 10 were classified as affected and 1 was classified as probably affected. Comparison of the number of mutant allele carriers with migraine (10 of 11) to the noncarriers with migraine (5 of 13) using a Fisher's exact test yielded a P value of 0.011. One would not expect association of CKI δ alleles with migraine at that high frequency by chance. Nonetheless, the fact that migraine occurred in some family members who did not express a mutant CKI δ allele suggests that each family may have other predispositions to the condition.

The association between two independent mutant CKI δ alleles and the migraine phenotype suggests that these mutations contribute to the pathogenesis of migraine. Further strengthening this argument are in vitro and in vivo mouse data that support a possible causative role of these variants in migraine.

Migraine is associated with increased sensitivity to all sensory modalities, and there is growing evidence that cutaneous allodynia is a

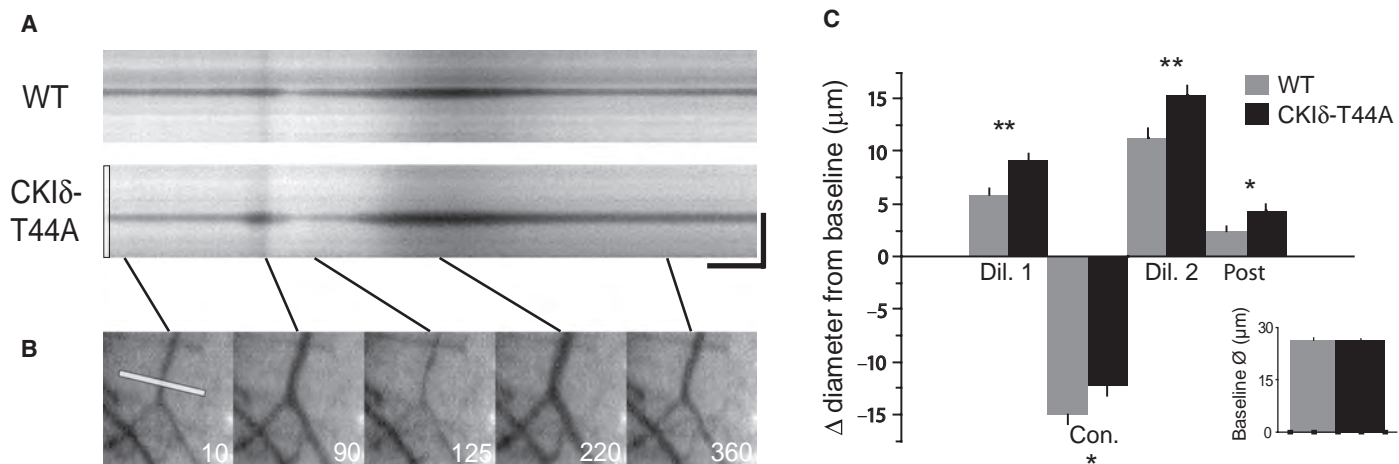


Fig. 5. Increased dilation and decreased constriction to CSD in CKIδ-T44A mice. (A) Arterial diameter changes in wild-type and CKIδ-T44A mice during CSD. Kymographs reveal arterial diameter changes during acute CSD. CSD is associated with arterial dilation that precedes the parenchymal CSD wavefront (Fig. 4A), followed by constriction, a second dilation, and a post-CSD dilation. Scale bar, 25 s, 125 μm. (B) The first image shows the line drawn across the artery at baseline that generates CKIδ-T44A kymograph. The following panels show each of the CSD-associated phases of arterial activity. (C) Mean arterial diameter changes. Despite nearly identical baseline

diameters (26.4 ± 0.7 versus 26.3 ± 0.5 μm; $P = 0.94$) in wild-type and CKIδ-T44A mice, dilation is more prominent and constriction is less marked in CKIδ-T44A versus wild-type arteries. Initial dilation (32.2 ± 0.9 μm versus 35.6 ± 0.7 μm; $P = 0.00008$), second dilation (37.7 ± 1.3 μm versus 41.7 ± 1.0 μm; $P = 0.002$), and post-CSD (28.8 ± 0.8 μm versus 30.7 ± 0.8 μm; $P = 0.01$) were all more dilated compared to baseline, and constriction was attenuated (11.4 ± 1.0 μm versus 14.1 ± 0.9 μm; $P = 0.02$, linear mixed-effects model with post hoc Tukey test) (wild type: $n = 6$ mice, 23 vessels, 67 measurements; CKIδ-T44A: $n = 9$ mice, 34 vessels, 110 measurements).

quantifiable marker of the disorder (33, 34). Hyperalgesia evoked by the migraine trigger NTG in mice is a model for this allodynia (19). Increased NTG-evoked mechanical and thermal hyperalgesia exhibited by CKIδ-T44A mice is consistent with a role for CKIδ in sensory hypersensitivity that is relevant to migraine. Increased NTG-induced activation of c-fos-positive nuclei in the trigeminal dorsal horn of CKIδ-T44A mice indicates increased activation of neurons associated with craniofacial pain in comparison to wild type (21, 35). CKIδ-T44A mice thus show both behavioral and functional anatomical evidence of altered craniofacial nociception.

CKIδ-T44A mice showed both a significant reduction in the threshold for evoking CSD and an increased number of CSD events elicited by continuous stimulation, consistent with increased cortical excitability that has been observed in patients with migraine. A similar susceptibility to CSD has been observed in mice expressing mutations in a P/Q-type Ca^{2+} channel and a Na^+/K^+ adenosine triphosphatase that are responsible for familial hemiplegic migraine types I and II, respectively (28, 36, 37). In addition to the difference in the susceptibility to CSD, the cortical vascular response to CSD is altered in CKIδ-T44A mice. Cortical and meningeal vessels contain trigeminal afferents and transmit nociceptive signals (21, 23); the altered vascular response to CSD in CKIδ-T44A mice could be relevant to migraine-related pain.

Increased spontaneous and evoked Ca^{2+} signaling in astrocytes from CKIδ-T44A mice compared to wild-type mice suggests that alteration in astrocyte signaling could be a mechanism by which the CKIδ-T44A mutation influences cortical excitability and associated vascular responses. Astrocyte Ca^{2+} waves are consistently observed in conjunction with CSD (29, 38, 39), and astrocyte signaling plays an important role in neurovascular coupling (40). Astrocytes release extracellular messengers including ATP and glutamate through undocked connexin channels

(hemichannels) (31, 41). Hypophosphorylation of connexin43 increases the proportion of connexin molecules existing as hemichannels in astrocytes compared with gap junctions, potentially leading to increased release of ATP and glutamate (30, 31, 41). We showed that connexin43 was hypophosphorylated by both mutant forms of CKIδ (Fig. 1) and that spontaneous and induced calcium signaling (Fig. 6), but not ATP release (fig. S5), was greater in cultured CKIδ-T44A astrocytes compared to wild type. Together, these findings suggest possible astrocytic mechanisms by which CKIδ mutations could predispose to migraine.

The relationship between the sleep phenotype and the migraine phenotype in individuals with alterations in the function of CKIδ is uncertain. Migraine can be modulated by sleep, and associations between migraine and a number of different sleep disorders have been reported (42). Mice expressing mutant alleles responsible for familial hemiplegic migraine type 1 have a circadian phenotype consisting of enhanced circadian phase resetting (43). Common cellular mechanisms may be involved in circadian function and episodic changes in brain excitability that cause migraine. On the other hand, migraine has not been reported as a part of the phenotype of other families with FASPS, indicating that the mechanisms underlying the migraine phenotype in families with CKIδ mutations may be distinct from those that cause alterations in sleep. Another interpretation is that CKIδ mutant families have a genetic background that enhances susceptibility to sleep phase-associated migraine triggers.

In summary, we have shown that mutations in CKIδ may contribute to the pathogenesis of human migraine and that mice expressing the CKIδ-T44A mutant enzyme show phenotypic features resembling those seen in the human disorder. The proteins encoded by all previously identified migraine genes are ion channels or pumps. CKIδ, in contrast, is an enzyme that modulates the functions of a variety of

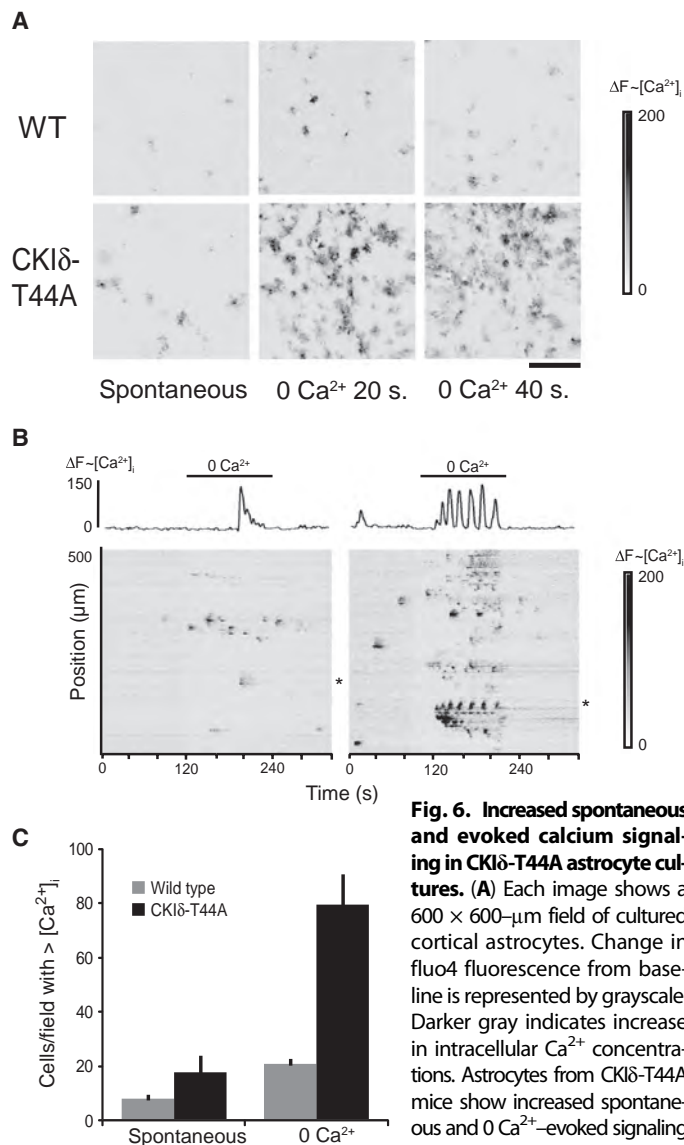


Fig. 6. Increased spontaneous and evoked calcium signaling in CKI δ -T44A astrocyte cultures. (A) Each image shows a 600 \times 600- μ m field of cultured cortical astrocytes. Change in fluo4 fluorescence from baseline is represented by grayscale. Darker gray indicates increase in intracellular Ca^{2+} concentrations. Astrocytes from CKI δ -T44A mice show increased spontaneous and 0 Ca^{2+} -evoked signaling compared with those from wild-type mice. Scale bar, 100 μ m. (B) Raster plots show pattern of Ca^{2+} signaling changes at baseline and upon exposure to 0 Ca^{2+} medium in wild-type (left) and CKI δ -T44A (right) astrocyte cultures. Traces (above raster plots) show the calcium response of a representative cell (indicated by asterisks) in wild-type and CKI δ -T44A cultures. (C) Average number of cells in a 600 \times 600- μ m field of cultured cortical astrocytes that show an increase in intracellular Ca^{2+} concentrations during 2 min under basal conditions (spontaneous) or during exposure to 0 Ca^{2+} medium (evoked). Error bars represent SEM. Significantly more astrocytes from CKI δ -T44A show spontaneous ($P = 0.0008$) and 0 Ca^{2+} -evoked increases in intracellular Ca^{2+} concentrations compared to wild-type mice ($P = 0.0006$) [$n = 16$ cultures, 200 cells per culture from eight different CKI δ -T44A mice, and 12 cultures, 200 cells per culture from six different wild-type mice (two-tailed t test for samples of unequal variance for each comparison)].

proteins, a subset of which could contribute to migraine pathogenesis. Identifying protein targets of CKI δ that are relevant to migraine may further elucidate the cellular signaling mechanisms underlying migraine, and identify new therapeutic approaches.

MATERIALS AND METHODS

Genetic association

The family members originally sought medical attention for symptoms of migraine, but during the course of evaluation, multiple individuals were also diagnosed with advanced sleep phase syndrome. Subjects signed consent or assent forms approved by the Institutional Review Boards at the University of California at San Francisco (UCSF) or the University of Utah. They then underwent physical and neurological examinations and structured interviews related to self-reported migraine traits and symptoms, circadian and sleep traits, and general health. Individuals were diagnosed with headache disorders according to the criteria of the ICHD-2. Individuals not meeting either “affected” or “unaffected” criteria were considered to be of “unknown” phenotype. Blood sample collection and DNA preparation were performed as previously described (14).

Polymerase chain reaction and Sanger sequencing of DNA samples

CKI δ was screened for mutations via Sanger sequencing of genomic DNA. Genomic information of CKI δ coding regions was obtained from Web site databases (National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov/> and UCSC Genome Bioinformatics: <http://genome.ucsc.edu/>) for primer design used for sequencing. Primers were designed outside of splice sites with the intent that intronic sequencing of at least 50 base pairs would flank each exon border. Twenty-five microliters of polymerase chain reactions (PCRs) was carried out per 100 ng of genomic DNA and 10 pmol of both forward and reverse primers. PCR procedures that lead to successful product amplification were as follows: 98°C, 30 s (98°C, 10 s; 60°C, 30 s; 72°C, 40 s) \times 35, 72°C, 10 min, and 4°C hold. PCR product purification was done with the PCR96 Cleanup Plate (Millipore) and then sequenced. The primer set used for sequenced exon 2 of CKI δ , which harbored the T44A mutation, was as follows: forward primer, 5'-tgctaggaaggagaacacatcc-3'; reverse primer, 5'-agctgtgactgcttgcagg-3'.

Protein purification and kinetic analysis

Subcloning, site-directed mutagenesis purification of enzymes, and kinase assays were performed as described (14). Briefly, reactions were conducted in 50 mM Hepes (pH 7.5), 1 mM dithiothreitol, and 1 mM $MgCl_2$ at 25°C, with catalytic amounts of CKI δ 317, CKI δ 317 T44A, or CKI δ 317 H46R (10 to 20 nM), 3000 Ci/mmol [γ - ^{32}P]ATP (1 to 500 μ M), and substrate (1 to 500 μ M, depending on the substrate, α -casein or PER2 peptide). Five microliters of the reaction mixture was spotted onto P81 Whatman paper and washed with 1% phosphoric acid. Radioisotope incorporation was assayed by exposure to phosphor-image screen and quantified on a Typhoon image scanner. Saturation experimental data were fit to the Michaelis-Menten equation with KaleidaGraph (Synergy Software), and parameters k_{cat} and K_m were determined.

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were cultured as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cotransfection of HEK293 cells was performed with 4 mg of total DNA with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and as described in the figure legend. Cell treatments were carried out as described in the figure

legends. Soluble cell extracts were made in radioimmunoprecipitation assay buffer (Sigma) with PhosSTOP and Complete tabs (Roche). Protein concentration was quantified by Bradford assay (Bio-Rad).

Immunoprecipitation and Western blotting

Immunoprecipitations were performed with anti-FLAG antibody-conjugated agarose resin (Sigma) as described by the manufacturer. Western blotting and PAGE were carried out with standard protocols.

Animal care and handling

All protocols were approved by the Institutional Animal Care and Use Committees at UCSF and UCLA. Experiments were conducted on CK1 δ -T44A line 827 (14) mice weighing between 20 and 30 g and their wild-type littermates. Animals were housed in temperature-controlled rooms on a 12-hour light-dark cycle. Experimenters were blind to the animal genotypes in all experiments.

Nociception experiments

NTG administration. A stock of NTG (5 mg/ml) (American Regent Inc.) dissolved in 30% alcohol, 30% propylene glycol, and water was further diluted fresh each day in 0.9% saline in a polypropylene tube to reach desired concentrations. Doses of 1, 3, 5, 7, or 10 mg/kg were administered intraperitoneally, with a polypropylene syringe to minimize the loss of NTG activity. Control mice received an intraperitoneal injection of 0.9% saline solution without NTG.

Behavioral assays. Animals were habituated to each testing apparatus for 60 min on the day before and again immediately before determination of baseline nociceptive thresholds. Each set of animals underwent nociception assay before and after application of a specific dose of NTG. Behavioral assays were conducted blind to the animal genotype.

Mechanical hypersensitivity testing. The von Frey (Semmes-Weinstein) monofilament method was used to test mechanical sensitivity. Animals were placed on a wire mesh stand, whereas calibrated Semmes-Weinstein monofilaments (North Coast Medical) were used for the up-and-down method of determining mechanical sensation of each hind paw before injection (baseline) and 1, 2, and 4 hours after NTG injection (44). Fourteen wild-type and 15 CK1 δ -T44A animals were tested. Left and right paws were scored separately for two readings per mouse per time point. Mice were given 1 hour in home cages with food and water immediately after the 2-hour measurement and were tested again 4 hours after NTG as approved by the Institutional Animal Care and Use Committees of responsible institutions.

Thermal hypersensitivity testing. To determine thermal hypersensitivity, the radiant heat (Hargreaves) assay focuses radiant light on the hind paw and measures the time in seconds until the mouse removes each hind paw from the source of heat (PAW Thermal Stimulator, UCSD Department of Anesthesia) (19, 45). Three determinations of response were taken for every time point with at least 2 min between each trial. The Hargreaves assay measured thermal nociceptive behaviors immediately before and 30, 60, 90, 120, and 240 min after injection of NTG. Mice were returned to home cages for 1 hour after the 2-hour measurement and were acclimated to the Hargreaves apparatus for 1 hour again before the 4-hour time point. Twenty-four CK1 δ -T44A mice were tested and 22 wild-type siblings were tested for doses of 1, 3, 5, 7, and 10 mg/kg. NTG thermal sensitivity experiments were repeated with one dose (5 mg/kg) at one time point (90 min) with 12 female CK1 δ -T44A and 12 wild-type siblings.

Rotarod testing. Nine mice of each genotype (CK1 δ -T44A mice and wild-type littermates) were tested for motor function and learning on the rotarod (46). The rotating rod attained a speed of 40 rpm over a period of 5 min. Three trials separated by 1 min each were completed in each set. Each animal underwent three sets of trials for a total of nine trials. Animals were given 15-min rest between each set of the tests.

Immunohistochemistry

CK1 δ -T44A and wild-type littermates were treated with intraperitoneal injection of NTG (5 mg/kg). Two hours after NTG, mice were deeply anesthetized with isoflurane and perfused intracardially with phosphate-buffered saline (PBS) and then 4% paraformaldehyde. Whole brain and spinal cord were fixed overnight in 4% paraformaldehyde and transferred to 30% sucrose in PBS at 4°C until sectioning. Transverse 35- μ m sections of frozen spinal cord and brain were cut. Free floating sections were incubated in PBS and 0.3% Triton X-100 and 5% normal goat serum (NGS-T) for 1 hour before incubation in primary anti-Fos (Oncogene Science) at a dilution of 1:40,000 in 5% NGS-T for 18 hours at 4°C. Sections were washed in PBS before incubation for 1 hour in biotinylated goat anti-rabbit antibody (Vector Laboratories), washed in PBS, and incubated in ExtrAvidin-Peroxidase (Sigma-Aldrich Biotechnology) for 1 hour, followed by detection of the peroxidase with 3,3'-diaminobenzidine (Sigma). Mounted and coverslipped sections were counted for Fos-reactive nuclei by a single blinded observer. Fos-positive cells were counted in TNC, C1 dorsal horn, or C2 dorsal horn in 6 to 10 sections per animal ($n = 6$ wild-type and 7 CK1 δ -T44A mice). Sections were excluded if the tissue was torn or damaged. Sections were visualized with an Olympus CX41 upright light microscope. Photographs were taken with a PixelINK PL-A662 and captured with the PixelINK Capture software. A two-tailed Student's t test was used to determine statistical significance.

CSD experiments

Anesthesia was induced (5%) and maintained (0.8 to 1.5%) with isoflurane. Temperature was maintained at $37 \pm 0.5^\circ\text{C}$ with a rectal temperature probe and homeothermic blanket. A pulse oximetry probe was placed on the right hindpaw (8600 V, Nonin), and hemoglobin saturation was maintained at $>95\%$. Anesthetic levels were adjusted to maintain the animal in burst suppression on field potential recordings, with an interburst interval of 4 to 9 s (47). At this anesthetic depth, the animal had no response to noxious stimulation. There were no significant differences in anesthetic level, temperature, hemoglobin saturation, or field potential burst duration or interburst interval between wild-type and CK1 δ -T44A mice ($P > 0.3$ for all comparisons, Student's t test).

Each animal was placed in a stereotaxic frame (Kopf Instruments), and the parietal skull was exposed. The skull was thinned to transparency, to boundaries 1 mm from temporal ridge, sagittal suture, bregma, and lambda. A glass recording electrode ($\sim 20\text{-}\mu\text{m}$ diameter, 1 M KCl fill solution, $\sim 0.5\text{-megohm}$ resistance) was advanced 550 μm into the cortex through a burr hole placed 0.5 mm anteromedial to lambda to collect field potentials. A silver-silver chloride ground wire was placed in the neck musculature. A second burr hole was placed 0.5 mm from the temporal ridge midway between bregma and lambda for CSD induction. A 34-gauge fused silica micropipette filled with 1 M KCl and attached to a pneumatic pico-pump (MicroFil and PV-820, WPI) was carefully apposed to the cortex in the second burr hole.

The animal was placed on the imaging stage of a custom microscope and rested under anesthesia for 1 hour before imaging. The cor-

tex was illuminated with white light (5500 K, 400- to 700-nm spectral range, Philips Lumileds). Reflected light was collected for OIS with a lens system consisting of two $f/0.95$ lenses connected front to front, focused on a high-sensitivity (0.00015 lux) 8-bit charge-coupled device camera (Watec 902K). Field of view was 4.2×3.2 mm, and pixel size was $6.6 \mu\text{m}$. Images were acquired at 2 Hz for 1 hour. Field potentials were acquired (bandpass, 0 to 1 kHz), amplified (A-M Systems 3000), digitized (at 1 kHz; PCI-6251, National Instruments), and recorded and synchronized with imaging data by a custom LabView Virtual Instrument (National Instruments).

CSD thresholding consisted of a series of pulses with progressively increasing pressure ejecting increasing volumes of KCl from the pipette (4 to 40 psi in 2-psi increments at 300-s intervals, corresponding to KCl volumes of 0.2 to 1.4 μl) until threshold for CSD was achieved (24). After determination of threshold, repeat inductions were performed at threshold levels every 10 min for a total of six KCl ejections. Volume ejected from the KCl pipette was verified before and after each experiment to ensure replicable thresholds. The KCl method of thresholding was used because it allowed focal administration of the KCl stimulus (electrical stimulation causes more diffuse activation and thus arterial dilation, which impedes measurement of CSD-associated vascular parameters). After thresholding, the animal was euthanized with 5% isoflurane followed by nitrogen asphyxia.

To test the rate of CSD in response to a constant stimulus, we positioned the KCl pipette just over the burr hole without touching the brain, and a 4-psi tonic output from the pneumatic pico-pump produced a constant flow of 1 M KCl into the burr hole during the course of an hour-long experiment.

Image analysis was performed with ImageJ [National Institutes of Health (NIH), Bethesda, MD] and IGOR Pro (WaveMetrics). Normalized reflectance images ($r/r_0 \times 100$) were generated by dividing each frame by an average of the first 10 frames of the experiment. To generate an optical trace of CSD, we traced a 100-pixel curved line region of interest (ROI) along the CSD wavefront at the point where it contacted the field potential electrode. Each pixel of the ROI generated a trace of the CSD waveform, synchronous with electrophysiological changes, which could be compared with the other traces in each experiment as well as with separate experiments. Surface arteries were identified by morphology and by reactivity to CSD, as previously described (27). To sample vessels in an unbiased manner, we divided each imaging field into quadrants. The arterial segment closest to the center of each quadrant was chosen for analysis, yielding four arterial ROIs per experiment. A 50-pixel (330- μm) line ROI was placed perpendicular to the long axis of the vessel. When plotted over the course of the experiment, this line ROI revealed the diameter of the vessel, which could be computed via pixel count (Threshold and Measure Functions, ImageJ). ROIs (6 \times 6 pixels) were placed immediately adjacent to the line ROIs that were used to measure arterial diameter. These allowed comparison of parenchymal and arterial changes in the same location.

OIS and field potential amplitudes and durations were measured after detecting the critical points in the respective intrinsic signal and field potential traces. This was done by computing the second derivative of each data set, allowing points of maximum change in slope to be identified. Five critical points were typically seen for intrinsic signal data: at the onset of changes, at the minimum of the first phase, at the maximum of the second phase, at the minimum of the third phase, and during a return to baseline levels (Fig. 3B). Three critical points

were found for field potential data: at the onset, maximum, and recovery of dc shift, respectively (Fig. 3B). OIS duration was calculated between the first and fourth critical points, and area under the curve was computed separately for each phase using its boundary critical points. dc shift duration was calculated between the first and third field potential critical points, and amplitude was calculated between the first and second critical points.

Calcium imaging of astrocytic cultures

Astrocyte cultures were prepared from 1-day postnatal mouse pups (31). Cortices were removed, mechanically dissociated, and filtered as above, but the cells were resuspended and plated on glass coverslips in DMEM/F12 medium supplemented with 5% FBS. At days 4 to 7 in culture, cells were mechanically shaken for 30 min per day, and non-adherent cells were removed. Cells were cultured for 14 days before experimentation.

Changes in intracellular calcium concentration were recorded with a custom confocal microscope as previously described (48). Briefly, cells were loaded with the calcium indicator fluo4 by bath exposure to 5 μM fluo4 acetomethoxy ester (Molecular Probes) for 30 min. Cells were washed repeatedly to remove excess indicator and then placed on the stage of a modified Nikon Diaphot microscope (Nikon Instruments). Excitation from a 475-nm diode laser was scanned via resonance mirrors to the coverslip through a 20×0.90 numerical aperture (Olympus) objective. Fluorescence emission was collected through a 535-nm bandpass filter to a photomultiplier tube (Hamamatsu), and images were acquired at 5 Hz at a pixel resolution of 940×720 by an image acquisition board (Raven, BitFlow) controlled by Video Savant (IO Industries) software. Solutions containing glutamate, *N*-methyl-D-aspartate (NMDA), or 0 Ca^{2+} were applied by bath exchange.

ROIs were placed on all cells in each microscopic field, and values for fluo4 fluorescence versus time were determined for each cell in the field for each experiment. Line traces and raster plots were generated from normalized images ($\Delta F/F_0$).

ATP release

Assays of released ATP were performed with methods similar to those previously described (31). Astrocyte cultures were prepared as above and grown in plastic dishes. To measure basal ATP release, we replaced the medium above the cultures with 0.7 ml of Hanks' balanced salt solution (HBSS) for 1 min. This medium was then removed, immediately frozen, and replaced with HBSS with no added Ca^{2+} and Mg^{2+} for 1 min, after which this medium was also collected and frozen. ATP assays were performed with the Enliten assay kit (Promega) with luminescence measurements performed with a Wallac Victor plate reader. The concentration of ATP released into normal medium in 1 min was subtracted from that released in low $\text{Ca}^{2+}/\text{Mg}^{2+}$ medium in 1 min to determine the amount of stimulated ATP release.

Statistical testing

Unless otherwise noted, one-way ANOVA or Student's *t* test was used, after determination that data sets were parametric. Post hoc testing was performed with Tukey test. Pearson's test was used to assess correlation between different CSD-associated measures. Statistical tests were implemented in IGOR Pro 6.0 (WaveMetrics). Unless otherwise noted, error bars denote SEM.

For nociception experiments, differences in response to mechanical and thermal stimulation were examined with a linear mixed-effects

model that accounted for both the correlation between repeated measurements taken across time on each mouse and the correlation between repeated measurements taken at each time point. The means, SEs, and differences of means reported in figures and in the text are based on the mixed model. Tests of the genotype-by-time interaction (P values in Fig. 2) assess the difference between CKI δ -T44A and wild type across the time spectrum. Analyses were conducted with SAS (version 9.3, SAS Institute Inc.).

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/5/183/183ra56/DC1

Fig. S1. Alignments for *Drosophila* Dbt and mouse (m) and human (h) CKI δ and CKI ϵ proteins.

Fig. S2. Motor function in wild-type and CKI δ -T44A mice.

Fig. S3. Gradient of CSD thresholds by sex and genotype.

Fig. S4. Scatterplot of CSD dc shift duration and threshold in all animals.

Fig. S5. Astrocyte ATP release evoked by low divalent cation solution.

Table S1. Mechanical sensitivity thresholds.

Table S2. Thermal responses.

Table S3. CSD-associated measures in CKI δ -T44A mice and wild-type littermates.

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Migraine: a brain state

Andrew Charles

Purpose of review

Migraine has traditionally been categorized as a pain disorder, focusing on headache as its central feature. This narrow view does not account for the complex array of premonitory and postdromal symptoms that occur in the hours before and after headache. This review outlines evidence that supports a broader view of migraine as a pathological brain state.

Recent findings

Studies of the clinical features of a migraine attack, in combination with imaging and electrophysiological studies, provide evidence that migraine involves widespread changes in brain function and connectivity. These changes parallel those seen in other brain states such as sleep. Neurochemical mediators, including adenosine, and nonsynaptic signalling mechanisms involving astrocytes may play a role in the migraine state.

Summary

Consideration of a migraine attack as a brain state provides an expanded framework for understanding all of its symptoms, and the underlying alterations in the activity of multiple brain networks. Mechanisms driving the transition to the migraine state may represent novel targets for acute and preventive therapies.

Keywords

adenosine, astrocytes, headache, resting states, sleep

INTRODUCTION

Brain states such as sleep, wakefulness and attention are characterized by consistent patterns of brain activity, the coordinated involvement of multiple brain regions or networks, and stereotyped associated behaviours or functional responses [1,2]. A migraine attack clearly includes all of these features, and therefore can be practically considered as a pathological brain state. What is the purpose of defining migraine in this way? Traditional definitions have naturally focused on headache, the most prominent symptom of migraine. But consideration of migraine simply as a 'trigemino-vascular' disorder or a pain disorder does not account for the complex constellation of migraine symptoms that precede, accompany or outlast headache. A more comprehensive definition of a migraine attack provides a context for a more definitive understanding of the disorder and for the development of novel and effective therapies. This review will summarize recent studies that support the concept of migraine as a brain state.

CLINICAL FEATURES OF THE MIGRAINE STATE

There are several parallels between the migraine state and the sleep state. Similar to sleep, there are

homeostatic, circadian and allostatic factors [3,4] that drive the initiation of a migraine attack. Food intake, fatigue, time of day, hormonal changes and stress are among the many variables that may be involved in the onset of migraine, just as they are with sleep. Also similar to the sleep state, multiple brain networks are either activated or inactivated during a migraine attack. Most studies of brain networks in migraine have focused on those involved in the perception and processing of pain [5[•],6[•],7[•]]. As discussed below, however, the clinical symptoms of migraine as well as electrophysiological and functional imaging studies indicate involvement of multiple brain networks in addition to those directly related to pain. One possibility is that migraine involves a disruption of the normal coordination between different brain networks that occurs during physiological brain states. With sleep, there

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KEY POINTS

- The diverse clinical features and widespread patterns of brain activity associated with a migraine attack are consistent with a pathological brain state.
- The migraine state may involve derangements of mechanisms involved in the maintenance of and switching between physiological brain states.
- Neurochemical mediators such as adenosine and nonsynaptic mechanisms involving astrocytes similar to those that initiate sleep may also play a role in the migraine state.

is coordinated activation of sleep-promoting networks and inhibition of wake-promoting networks [3]. Similarly, in most normal physiological states (with the exception of rapid eye movement sleep), arousal is coordinated with awareness [8]. By contrast, in some pathological states such as the vegetative state and sleepwalking, there may be relatively greater arousal than awareness. The migraine state represents the opposite situation, wherein decreased arousal (as indicated by the symptoms of yawning, fatigue and somnolence) is accompanied by heightened awareness (as indicated by increased sensitivity to light, sound, smell and touch). This clinical picture suggests that in addition to activation of pain networks, the migraine state involves a derangement of the normal physiological relationship between other brain networks such as those regulating sleep, wakefulness, awareness and arousal.

Hypotheses of migraine pathogenesis have typically focused on a primary region of initiation such as spreading depression in the cortex or a 'migraine generator' in the brainstem. But the temporal progression of a migraine attack indicates simultaneous changes in the function of multiple brain regions, and it is not clear that there is a single anatomical region wherein migraine begins in all patients. Premonitory symptoms occurring up to hours before headache, including neck pain, fatigue, mood change, yawning, polyuria and light sensitivity, may last into the headache phase of an attack and extend into the postdrome lasting hours following resolution of headache [9–12]. Similarly, prospective recording of symptoms indicates that headache, light sensitivity and nausea commonly occur at the same time as aura [13[¶]]. Symptoms that persist after resolution of the headache include neck pain, mood change, fatigue and asthenia [11,14,15]. This overlapping pattern of symptoms is consistent with parallel alteration in the activity of different anatomical regions rather than a linear cascade of changes with one leading to the next.

IMAGING STUDIES INDICATE WIDESPREAD CHANGES IN BRAIN FUNCTION DURING MIGRAINE

The initial functional imaging studies of migraine highlighted changes in the activity of brainstem and cortex during a migraine attack [16–18]. More recent studies have also shown concomitant changes in the activity of other brain regions. Activation of the hypothalamus has been shown to occur during migraine [19], and this change in hypothalamic activity may be particularly important in the premonitory phase of migraine that precedes headache [20]. Such alteration of hypothalamic function could explain a number of common migraine symptoms including changes in appetite, mood and wakefulness that occur before a headache begins. Functional MRI (fMRI) studies have also recently demonstrated changes in thalamic activation during a migraine attack that are correlated with allodynia [21]. Thalamic activation could play an important role in the sensitization to all sensory modalities that occurs during a migraine attack. Previous PET and MRI studies showed dramatic changes in the activity of the occipital cortex associated with migraine visual aura [17,18], but more recent studies have also demonstrated increased activation of the occipital cortex in response to visual stimuli during a migraine attack correlated with photophobia in patients without visual aura [22^{¶¶}]. Interestingly, this increased occipital cortical sensitivity persisted after relief of headache. Light sensitivity also commonly precedes headache in a migraine attack [12], suggesting that increased excitability of the visual system is a feature of the migraine state that can both precede and outlast headache.

fMRI techniques have been used extensively in recent studies to identify brain network activity that is characteristic of specific brain states [1,2]. This fMRI approach relies on the identification of brain regions that display correlated low frequency (<0.1 Hz) oscillations in blood oxygen level dependent signal, indicative of functional connectivity between these regions. 'Resting states' are defined as those that occur in the absence of external stimulation. The most commonly studied resting state network is the default mode network – brain regions that are more active under conditions of rest and less active during cognitive activation, including the posterior cingulate cortex, anterior cingulate cortex and medial prefrontal cortex among others [1,2,7^{¶¶}]. It is likely that the migraine state will show characteristic changes in resting state activity, although no studies to date have reported such changes during a migraine attack. Structural and fMRI studies have demonstrated interictal alterations in connectivity

in individuals with migraine, particularly in brain networks involved in pain processing [5[■],7[■],23,24,25[■],26]. Interictal changes in the activity of networks involved in executive function [27[■]] and visual motion processing [23,28] have also been shown in migraine patients. These changes in connectivity are typically believed to be a consequence of migraine, as they are correlated with migraine frequency and duration. It is also possible, however, that these interictal structural and functional alterations contribute to disruptions in normal brain connectivity that occur *during* the migraine state.

ELECTROPHYSIOLOGICAL FEATURES OF THE MIGRAINE STATE

Brain states may be characterized by specific patterns of electrophysiological activity as measured by electroencephalography, magnetoencephalography or evoked potentials. Although much less pronounced than those observed in other brain states, electrophysiological changes indicating extensive changes in brain activity do occur before and during a migraine attack. Quantitative electroencephalogram (EEG) studies have been reported to show increased slowing in the theta range and increased asymmetry of EEG signal in the hours preceding a migraine attack and during the attack [29[■]]. Evoked potential studies also show characteristic changes in activity with migraine. Somatosensory evoked potentials have a high frequency component that is believed to reflect activity in a thalamo-cortical circuit. These high frequency oscillations (HFOs) are reduced in amplitude and area between attacks, and are normalized during attacks [30]. Recent studies have found that changes in HFOs are correlated with clinical fluctuations in migraine [31[■]], and that HFOs can be normalized by repetitive transcranial magnetic stimulation (rTMS) [32[■]]. These observations suggest that changes in the activity of brain-stem arousal networks and related thalamocortical networks are involved in the initiation of migraine and provide further support for the concept of a migraine attack as a brain state.

NEUROCHEMICAL MEDIATORS OF THE MIGRAINE STATE

A number of neurochemical mediators have been implicated in the transition from the wake to the sleep state. The transition to the migraine state may similarly involve changes in the levels of a variety of neurochemical mediators. Parallels between premonitory symptoms in migraine and those evoked by administration of dopamine receptor agonists have led to the hypothesis that dopamine may be one of

these mediators [33]. Orexins have also been proposed as potential mediators of both migraine and sleep [34]. Other interesting candidates for mediators of the migraine state that have been studied extensively in the context of sleep are the purines (ATP and adenosine) [35–37]. Extracellular adenosine levels increase during wakefulness, and a longstanding hypothesis has been that this increased adenosine plays a role in sleep induction by activation of receptors that inhibit neuronal activity [37]. An appealing aspect of this hypothesis is that it links cellular energy metabolism (adenosine is a product of ATP breakdown) and the drive to sleep, thus representing a homeostatic mechanism that may act in parallel with the hypothalamic circadian clock. ATP levels have been reported to rise during early stages of sleep in animals, supporting the hypothesis that one function of sleep is to restore a balance between ATP and adenosine [38]. Interictal magnetic resonance spectroscopy studies show a decrease in brain phosphocreatine content, and a decrease in occipital lobe ATP interictally in some migraine patients [39[■]], consistent with an alteration in ATP and other high energy substrates in migraine. Although there is no direct evidence for a role for adenosine in migraine, the migraine therapeutic effects of caffeine, a nonselective adenosine receptor antagonist, provide support for such a role. It is also interesting to speculate that adenosine could play a role in the fatigue, yawning and asthenia associated with migraine. The development of selective adenosine receptor antagonists for other indications may provide an opportunity for more definitive investigation of a hypothesized role for adenosine in the migraine state.

A ROLE FOR ASTROCYTES?

Diffuse changes in the activity of multiple brain regions suggest that the migraine state may not only involve traditional neuronal circuits but also non-synaptic mechanisms that include glial cells. There is growing evidence that astrocytes play a significant role in the homeostatic mechanisms regulating the sleep/wake states, and it is reasonable to hypothesize that they may also play a role in the migraine state. Animal studies indicate that a significant proportion of the adenosine that accumulates during wakefulness may be derived from astrocytes, and this astrocyte-derived adenosine can alter synaptic function by activation of A1 receptors [40[■]]. Recent in-vivo studies in mice also indicate that multiple general anaesthetics selectively inhibit calcium signalling in astrocytes, raising the possibility that suppression of astrocyte activity is a mechanism of general anaesthesia [41[■]]. Genes expressed primarily in astrocytes

have also been implicated in migraine with and without aura, as well as in familial hemiplegic migraine, lending support to the concept that astrocytes may contribute to the diffuse changes in brain function that occur during a migraine attack [42,43[■]]. Astrocytes show propagated waves of activity whose temporal and spatial characteristics are remarkably similar to those of cortical spreading depression, and we have suggested that these waves could be involved in propagated changes in brain activity and vascular function that are observed in patients with migraine [44]. Astrocytes are known to mediate bidirectional signalling between neurons and the vasculature, and astrocyte regulation of vascular function may be influenced by metabolic factors including adenosine levels [45]. Astrocytes could therefore play a significant role in the vascular changes and neurovascular uncoupling [46] that are observed as a part of the migraine state.

CONCLUSION

Conceptualizing a migraine attack as a brain state facilitates consideration of the entire array of migraine symptoms, as well as its complex neurochemical, cellular and anatomical substrates. Expanding the definitions of migraine beyond the traditional narrow focus on aura and headache creates opportunities for new experimental paradigms to study the disorder, and new therapeutic approaches. Comparison of the similarities and differences between migraine and other brain states, enabled by advances in imaging and physiological recording techniques, is likely to provide critical new insight into the causes of migraine and how to more effectively treat it.

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Conflicts of interest

There are no conflicts of interest.

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Characterization of a novel model of chronic migraine



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ABSTRACT

Chronic migraine is a disabling condition that affects hundreds of millions of individuals worldwide. The development of novel migraine treatments has been slow, in part as a result of a lack of predictive animal models. We have developed a new model of chronic migraine involving the use of nitroglycerin (NTG), a known migraine trigger in humans. Chronic intermittent administration of NTG to mice resulted in acute mechanical hyperalgesia with each exposure as well as a progressive and sustained basal hyperalgesia. This chronic basal hyperalgesia occurred in a dose-dependent fashion and persisted for days after cessation of NTG administration. NTG-evoked hyperalgesia was exacerbated by the phosphodiesterase 5 inhibitor sildenafil, also a human migraine trigger, consistent with nitric oxide as a primary mediator of this hyperalgesia. The acute but not the chronic basal hyperalgesia was significantly reduced by the acute migraine therapy sumatriptan, whereas both the acute and chronic hyperalgesia was significantly attenuated by the migraine preventive therapy topiramate. Chronic NTG-induced hyperalgesia is a mouse model that may be useful for the study of mechanisms underlying progression of migraine from an episodic to a chronic disorder, and for the identification and characterization of novel acute and preventive migraine therapies.

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1. Introduction

Migraine is one of the most common disorders affecting the general population, resulting in a staggering amount of episodic disability and lost productivity worldwide. For a significant percentage of patients, it results in chronic disability [4,28,29,32]. Despite the extraordinarily high prevalence of migraine, our understanding of its pathophysiology is incomplete. Moreover, although there has been significant progress in the acute treatment of migraine attacks, the ability to treat frequent and chronically disabling migraine remains severely limited. There continue to be millions of individuals for whom currently available migraine therapies are either ineffective or poorly tolerated [4,29].

A significant obstacle to the identification of new migraine therapies has been the lack of predictive animal models. There are a number of promising models for acute migraine attack [2,3,12,25], but it has been particularly difficult to study the pro-

gression of migraine from an episodic to a chronic disorder. One approach to modeling acute migraine is the quantification of increased sensory sensitivity in response to known migraine triggers. Nitroglycerin (NTG) reliably triggers headache in normal subjects, and it triggers migraine without aura in migraine-susceptible patients [1,9,17,24]. NTG-evoked migraine is a commonly used experimental paradigm in humans (for review, see [15,24,23]). NTG-evoked hyperalgesia in rodents has been developed as a model for sensory hypersensitivity associated with migraine [3,20]. Acute NTG was previously shown to produce thermal and mechanical allodynia in mice that was reversed by the antimigraine therapy sumatriptan [3]. In addition, in a transgenic mouse model of familial migraine, animals expressing a human migraine gene showed an even greater sensitivity to NTG-evoked hyperalgesia [6]. Further, NTG has also been shown to produce light-aversive behavior [20] and increased meningeal blood flow in mice [13,20]. Taken together, these results indicate that the effects of NTG may effectively model migrainelike symptoms in rodents. Here we have extended the NTG-evoked hypersensitivity assays to model the progression of migraine from an acute to a chronic state.

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2. Materials and methods

2.1. Animals

Subjects were male and female C57BL6/J mice, weighing 20 to 30 g. Animals were housed in a 12-h light–dark cycle, and food was available ad libitum. All experiments were approved by the University of California Los Angeles Office of Animal Research and the Animal Care Committee at the University of Illinois at Chicago, in accordance with AALAC guidelines. These experiments adhered to the guidelines of the Committee for Research and Ethical Issues of IASP [33].

2.2. Drug administration

NTG was prepared from a stock solution of 5.0 mg/mL NTG in 30% alcohol, 30% propylene glycol, and water (American Regent). NTG was freshly diluted in 0.9% saline to a dose of 10 mg/kg. The vehicle control used in these experiments was 0.9% saline. We found that there was no significant difference in mechanical thresholds between those observed when 0.9% saline was used vs those observed with 6% propylene glycol, 6% alcohol, and 0.9% saline. All injections were administered as a 10 mL/kg volume. Unless otherwise noted, animals were tested for baseline responses immediately before intraperitoneal (i.p.) injection with NTG. Animals were injected with subsequent drugs (i.p. unless otherwise noted) at 1 h 15 min after NTG injection, then were tested for mechanical or thermal sensitivity 45 min later (2 h after NTG). For chronic experiments, testing occurred every second day over 9 days (5 test days total). For the topiramate experiment, mice were injected with topiramate or vehicle every day for 11 days. On days 3, 5, 7, 9, and 11 of this treatment, basal mechanical sensitivity was determined, and mice received an injection of NTG or vehicle followed by an injection of topiramate/vehicle, and postdrug responses were determined 2 h later. For experiments testing localized intrathecal injections of sumatriptan into the central nervous system, drug (0.06 µg) or 0.9% saline was injected in a final volume of 5.0 µL [22]. Intrathecal injections were performed with a 30-gauge, ½-inch needle at the L4–5 lumbar interspace on lightly restrained, unanesthetized mice.

2.3. CFA-induced inflammatory pain

Inflammatory pain was induced by injecting Complete Freund's Adjuvant (CFA; 1 mg *Mycobacterium tuberculosis* [H37Ra, ATCC 25177]/mL of emulsion in 85% paraffin oil and 15% mannide monooleate; Sigma) into the paw. Before the injection of CFA, baseline mechanical responses were determined. Inflammation was induced by injecting 15 µL of CFA into the plantar surface of the paw, and animals were subsequently tested 72 h later.

2.4. Sensory sensitivity testing

To determine mechanical sensitivity, the threshold for responses to punctate mechanical stimuli (mechanical hyperalgesia) was tested according to the up-and-down method [8]. In brief, the plantar surface of the animal hind paw was stimulated with a series of 8 von Frey filaments (bending force ranging from 0.01 to 2 g). A response was defined as a licking, lifting or shaking of the paw upon stimulation. The first filament tested was 0.4 g. In the absence of a response a heavier filament (up) was tried, and in the presence of a response a lighter filament (down) was tested. This pattern was followed for a maximum of 4 filaments after the first response.

2.5. Statistical analysis

Data are expressed as mean ± SEM. All statistical analyses were performed by Sigmapstat software. For all acute pain experiments,

1-way analyses of variance (ANOVAs) were performed, and for chronic pain experiments, a 2-way repeated measures ANOVA was performed. Unless otherwise noted, all experiments were further analyzed by Holm–Sidak post hoc analysis. A significance level of $P < .05$ was used.

3. Results

3.1. Repeated administration of systemic NTG produces acute and chronic hyperalgesia

Acute administration of NTG has been shown previously to evoke severe mechanical and thermal hyperalgesia [3]. To model the progression to chronic migraine, we administered varying doses of NTG every second day for 9 days, resulting in a total of 5 NTG injection/test days. Mechanical thresholds were tested before and 2 h after NTG administration on each test day. Repetitive intermittent NTG administration over 9 days produced a significant time and dose-dependent chronic basal mechanical hyperalgesia as assessed by testing before each administration of NTG (Fig. 1A). In addition, NTG evoked significant acute mechanical hyperalgesia in a dose-dependent manner on each test day (Fig. 1B). The greatest decrease in basal and posttreatment responses were observed with 10 mg/kg, the dose of NTG previously characterized in an acute study [3]. We chose to further characterize this dose (Fig. 2). Progressively increasing basal hyperalgesia was observed with repeated administration of 10 mg/kg NTG (Fig. 2A). After the final treatment day (day 9), mechanical responses in mice were assessed daily to determine the recovery time for this basal hypersensitivity. Sensory responses returned to the level of naïve mechanical thresholds (day 1) by day 7 after NTG (Fig. 2A). Female mice showed greater basal hyperalgesia in response to chronic NTG, and unlike males, basal sensitization was observed after a single NTG injection (Fig. 2B). In addition, significant acute/post-treatment hyperalgesia was again observed with each administration (Fig. 2C). To determine whether the associative learning of repeated testing contributed to the progression of basal hyperalgesia, we examined basal mechanical thresholds in mice that had received identical intermittent NTG treatment over 9 days, but were only tested on the first day and on day 9 (novice). These novice mice did not show significantly different hyperalgesia on the final day of NTG (novice, 0.15 ± 0.06 vs repeatedly tested, 0.05 ± 0.03).

3.2. The phosphodiesterase 5 inhibitor sildenafil increases acute and chronic mechanical hyperalgesia

To investigate the role of cGMP in NTG-induced basal and evoked hyperalgesia we examined the effects of the phosphodiesterase 5 inhibitor sildenafil, which increases levels of cGMP and augments the effects of nitric oxide as an activator of guanylate cyclase. We treated mice every second day for 9 days (5 test days) with a low dose of NTG (1 mg/kg i.p.), and sildenafil (3 mg/kg i.p., Fig. 3). On each day of testing, chronic treatment with this low dose of NTG alone did not result in chronic basal hyperalgesia (Fig. 3A). However, the combination of this low dose NTG with sildenafil produced significant basal hypersensitivity. Interestingly, by the final day of testing, sildenafil alone had also produced a significant decrease in basal mechanical threshold (Fig. 3A). In addition, the combination of these 2 compounds produced a significantly greater acute hyperalgesia (Fig. 3B), than either compound alone. These results indicate changes in cGMP levels play a role in the hyperalgesia induced by chronic intermittent NTG treatment.

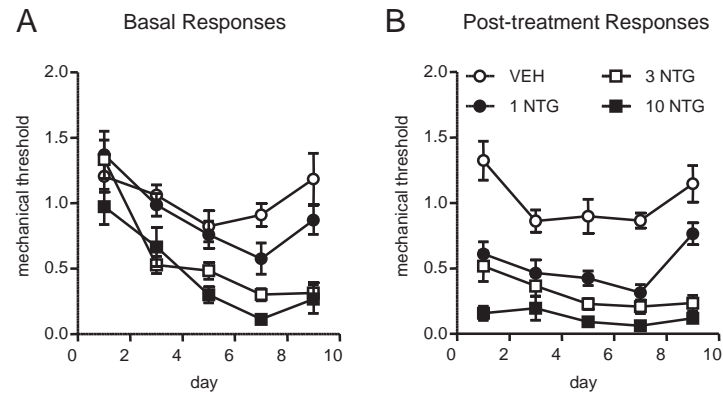


Fig. 1. Chronic NTG evokes and sustains mechanical hyperalgesia in a dose-dependent manner. C57Bl/6j mice were treated every second day with varying doses of NTG (0–10 mg/kg, i.p.) for 9 days. (A) Basal mechanical responses, as assessed before vehicle or NTG administration, significantly decreased in the 3 and 10 mg/kg NTG group during the treatment period; $n = 8/\text{group}$, $P < .001$ effect of dose and time 2-way repeated measures (RM) ANOVA; the 3 and 10 mg/kg doses were significantly different from vehicle and 1 mg/kg ($P < .001$). (B) Increasing doses of NTG produced increasing levels of mechanical hyperalgesia, in mice tested 2 h after NTG or vehicle administration; $n = 8/\text{group}$, 2-way RM ANOVA with Holm-Sidak multiple comparisons. Each dose was significantly different from vehicle ($P < .001$), and each dose was also significantly different from each other ($P < .01$). Chronic NTG in rodents produces a dose-dependent persistent hypersensitivity.

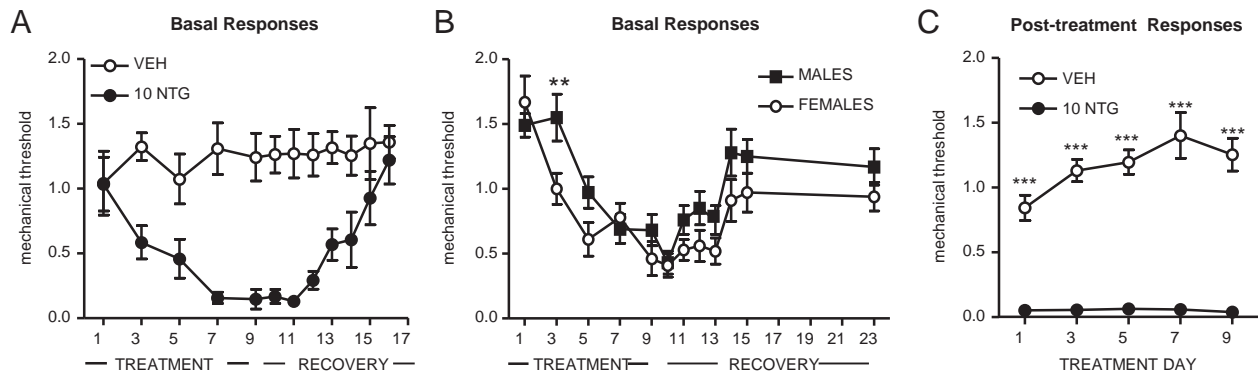


Fig. 2. Chronic NTG evokes severe and sustained mechanical hyperalgesia. C57Bl/6j mice were treated every second day with vehicle (VEH) or NTG (10 mg/kg, i.p.) for 9 days. (A) Basal mechanical responses, as assessed before vehicle or NTG administration, significantly decreased in the NTG group during the treatment period, and took 7 days to recover after the final NTG injection; $n = 7\text{--}8/\text{group}$. (B) Females developed basal mechanical hyperalgesia more quickly than males after chronic NTG treatment; $n = 12/\text{group}$, $**P < .01$ 2-way repeated measures (RM) ANOVA. (C) NTG consistently produced severe mechanical hyperalgesia, in mice tested 2 h after NTG or vehicle administration; $n = 7\text{--}8/\text{group}$, $***P < .001$ 2-way RM ANOVA. Chronic NTG in rodents appears to model the persistent hypersensitivity observed in migraine patients.

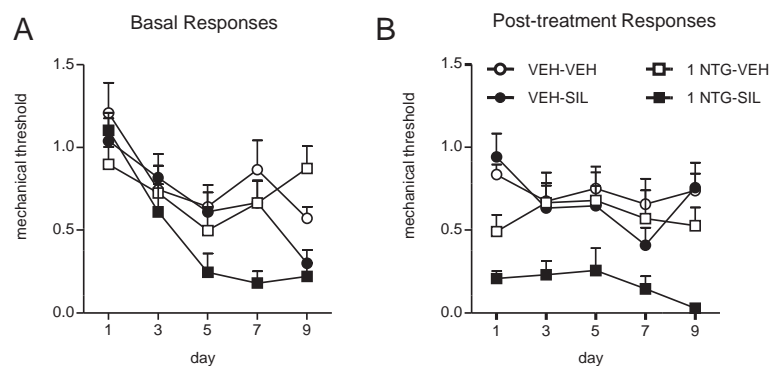


Fig. 3. Sildenafil, a phosphodiesterase 5 inhibitor, potentiates hyperalgesia induced by a low dose of NTG. C57Bl/6j mice were treated every second day with vehicle (VEH) or a low dose of NTG (1 mg/kg, i.p.) for 9 days. (A) Basal mechanical responses were assessed before NTG administration, $n = 8/\text{group}$, $P < .05$ effect of drug and $P < .001$ effect of time and interaction, 2-way repeated measures (RM) ANOVA; NTG-SIL mice were significantly different from all other groups ($P < .01$). (B) Mice were injected i.p. with either vehicle (VEH) or 3 mg/kg sildenafil (SIL) 1 h 15 min after NTG/VEH administration, and tested 45 min later (2 h after NTG); $n = 8/\text{group}$, $P < .001$ effect of drug, 2-way RM ANOVA; NTG-SIL mice were significantly different from all other groups ($P < .001$). Animals treated with the combination of NTG and sildenafil showed significantly reduced basal and posttreatment responses.

3.3. Sumatriptan alleviates acute NTG-evoked, but not chronic, basal hyperalgesia

We also investigated the effects of the migraine-selective acute therapy sumatriptan in the chronic NTG model. Consistent with previous studies [3], we found that sumatriptan (600 µg/kg, i.p.) reversed the acute hyperalgesia evoked by NTG injection. Systemic Sumatriptan continued to effectively ameliorate acute NTG-evoked hyperalgesia with each injection over 9 days. It did not, however, alter the chronic basal hyperalgesia that occurred over time with chronic intermittent NTG exposure (Fig. 4A, B). These results indicate that while sumatriptan can reverse the acute effects of NTG, it does not reduce the progression of hyperalgesia that occurs with repeated exposure. To address the possibility that sumatriptan non-specifically inhibited nociception, we tested this same dose of sumatriptan within the CFA model of peripheral inflammatory pain. Sumatriptan (600 µg/kg i.p.) was ineffective at reversing CFA-induced mechanical hyperalgesia (Fig. 4C). To investigate the possibility that sumatriptan was acting to inhibit NTG-evoked mechanical hyperalgesia at the spinal level, we injected sumatriptan 0.06 µg (a dose previously shown to have antihyperalgesic effects in certain peripheral and visceral pain models [22]) or vehicle intrathecally into the spinal L4–5 region. In contrast to systemic intraperitoneal administration, intrathecal injection of sumatriptan did not result in any inhibition of NTG-evoked hyperalgesia (Fig. 4D).

3.4. Topiramate inhibited NTG-induced chronic basal hyperalgesia

We then examined the effect of topiramate, a migraine preventive therapy, on acute and chronic basal hyperalgesia evoked by

NTG. Mice were treated once daily with topiramate (30 mg/kg, i.p.) for 11 days. On days 3, 5, 7, 9, and 11 basal and acute NTG-evoked mechanical responses were determined. Daily treatment with topiramate significantly inhibited the development of chronic basal hyperalgesia induced by chronic intermittent NTG treatment (Fig. 5A), and it also reduced acute NTG-evoked hyperalgesia (Fig. 5B).

4. Discussion

Our results indicate that chronic intermittent treatment with NTG produces an acute and chronic hypersensitivity which represents a translationally significant model of chronic migraine. Repeated injection of NTG evoked mechanical hyperalgesia, which was blocked by the antimigraine medication sumatriptan. We also found that chronic intermittent treatment with NTG produced a severe and long-lasting basal hyperalgesia, which was slightly more pronounced in female mice. In addition, this basal hypersensitivity was alleviated by treatment with the migraine prophylactic, topiramate. Furthermore, the effects of concomitant treatment with another human migraine trigger, sildenafil, confirmed that this basal hypersensitivity was mediated by increased levels of cGMP.

Several lines of evidence indicate that NTG treatment in rodents could be a predictive model of migraine in humans. NTG is a reliable trigger of migraine in susceptible patients [9,17,24]. Interestingly, migraine attacks do not occur immediately after NTG administration with the vasodilatory effects of the drug, but rather after 2 to 6 h [9,17,24], consistent with a delayed response to nitric

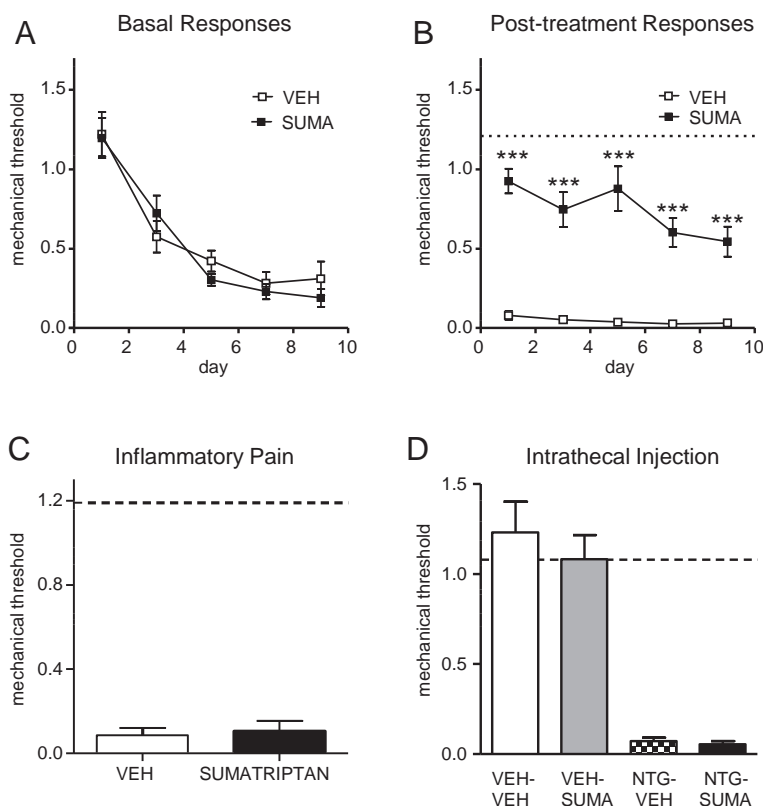


Fig. 4. Chronic NTG-evoked hyperalgesia is attenuated by systemic administration of sumatriptan. C57Bl/6J mice were treated every second day with NTG (10 mg/kg, i.p.) for 9 days. (A, B) Basal mechanical responses were assessed before NTG administration, and significantly and progressively decreased after the first treatment day. NTG injection produced further mechanical hyperalgesia (NTG-VEH), which was significantly attenuated by sumatriptan (B, 600 µg/kg, i.p.). Dashed line represents basal responses on day 1, $n = 11$ /group, $***P < .001$, 2-way repeated measures (RM) ANOVA. (C) Intraplantar injection of CFA into the hind paw produced significant hyperalgesia which was not inhibited by sumatriptan (600 µg/kg, i.p.). Dashed line represents baseline naïve mechanical responses before CFA injection, $n = 6$ /group. (D) Mice were injected with vehicle (VEH) or NTG (10 mg/kg, i.p.) and after 1 h 30 min injected intrathecally with sumatriptan (0.06 µg in 5 µL) and tested 30 min later. Dashed line represents basal responses before NTG injection. Pain produced by chronic NTG is alleviated by a systemically administered prototypic antimigraine treatment.

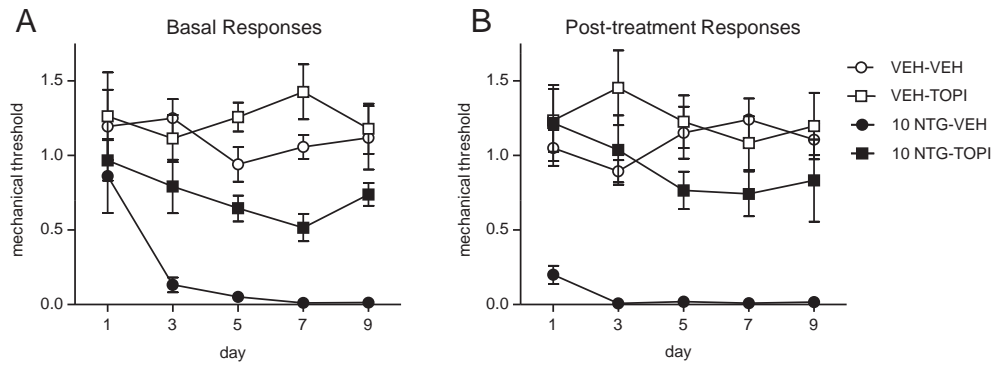


Fig. 5. Chronic treatment with the migraine prophylactic, topiramate, significantly attenuated NTG-induced basal and posttreatment hyperalgesia. C57Bl/6J mice were treated with vehicle (VEH) or topiramate (30 mg/kg i.p.) daily for 11 days. On days 3, 5, 7, 9, and 11 baseline responses were determined (A), and mice were injected with either vehicle (VEH) or NTG (10 mg/kg, i.p.). (B) Posttreatment responses were assessed 2 h after NTG administration (1 h 15 min post-topiramate); $n = 8/\text{group}$, $P < .001$ effect of NTG, 2-way RM ANOVA. Compared to the NTG-VEH group, NTG-TOPI showed significantly higher basal posttreatment response ($P < .001$). Both basal and acute hyperalgesia induced by chronic NTG can be alleviated by a well-characterized migraine preventative therapy.

oxide not mediated by vasodilation [27]. In mice, the timing of NTG-induced hyperalgesia also shows a delayed response, similar to the time course of headache observed in humans [3]. Systemic administration of NTG has been reported to cause cellular activation in nociceptive pathways, including trigeminal nociceptive pathways [3,26,30,31], providing additional support for relevance to migraine. In addition, NTG has been shown previously to cause light allodynia [20], and to increase meningeal blood flow [13,20] in mice, two hallmarks of migraine. Further, the amelioration of NTG-evoked hyperalgesia by the prototypic antimigraine medication sumatriptan also validates this model for the study of migraine mechanisms. Additionally, acute NTG-induced hyperalgesia has recently been shown to be increased in mice expressing a gene associated with familial migraine [6].

Migraine typically starts as an episodic disorder, but commonly progresses to a frequent or even daily condition [5]. The pathophysiological mechanisms underlying this change are poorly understood. We have extended the previously reported studies on acute NTG-evoked hyperalgesia to show that repetitive intermittent administration of NTG produces a progressive and sustained basal hyperalgesia that persists for days after the last exposure. These results are consistent with clinical observations of patients with chronic migraine in whom allodynia may occur both between and during migraine attacks. In addition, women are far more likely to suffer from chronic migraine than men [32], and our results suggest that female mice may be more sensitive to chronic NTG. Previous studies have also shown that there are estrogen-related discrepancies in the brain regions activated with NTG administration in rats [14], indicating that the chronic NTG model could potentially be a tool to specifically study sex differences in migraine.

The enhancement of the effects of NTG by sildenafil, also an established human migraine trigger [23,18], provides evidence that nitric oxide-mediated increases in cGMP play a primary role in the hyperalgesia evoked by NTG. We found that by the final test day, sildenafil also independently produced basal hyperalgesia with repeated intermittent dosing, suggesting that potentiation of the endogenous activity of guanylate cyclase may be a mechanism for producing chronic hyperalgesia. Drugs that target different aspects of the nitric oxide-cGMP pathway are currently being developed for the treatment of headache [23], and our preclinical data further support the role of this signaling cascade in the development of chronic migraine.

We found that sumatriptan inhibits acute hyperalgesia in response to NTG, similar to the results of previous rodent [3] and human [16] studies. We also found that sumatriptan continued to

have this effect with each NTG exposure over time, but that it did not inhibit the resulting progressive chronic basal hyperalgesia. This result is consistent with the observation that while sumatriptan can be consistently effective as an acute migraine therapy, its use does not prevent progression of migraine over time [4]. Since this model tests hyperalgesia in the hind paw, it is possible that the effects of sumatriptan could be mediated by spinal mechanisms. However, we found that intrathecal administration of sumatriptan did not inhibit NTG-evoked mechanical hyperalgesia, in contrast to systemically administered sumatriptan, indicating that within this model sumatriptan is not acting solely through the lumbar spinal cord. In addition, groups studying the dural inflammation model of migraine have also found that inflammatory mediators applied to the dura [12] or dural TRPA1 activation [11] produced mechanical hyperalgesia in both facial and hind paw regions, likely through brainstem relays. Cutaneous allodynia after NTG likely reflects the development of central sensitization which is observed during primary headache, which may be mediated through sensitization of neurons within the thalamus [7].

Topiramate inhibited acute NTG-evoked hyperalgesia, as well as chronic basal hyperalgesia, consistent with its actions as a prophylactic migraine therapy. Topiramate has multiple mechanisms of actions that could be involved in its therapeutic efficacy for migraine, including inhibition of voltage gated sodium channels, enhancement of GABAergic signaling, inhibition of glutamatergic signaling, and inhibition of carbonic anhydrase [21]. It has been proven to be an effective preventive therapy even in the setting of chronic migraine [10]. Moreover, on the basis of limited studies, topiramate has also been suggested to inhibit the progression of migraine to a chronic disorder in patients [19].

In this study we used a dose of NTG (10 mg/kg), which is substantially higher than the equivalent dose used to provoke migraine in humans [24]. It is therefore possible that this dose of NTG in mice could have additional effects apart from those associated with migraine induction in humans. The significant inhibition of NTG-induced hyperalgesia by systemic sumatriptan and topiramate, however, indicates that even if this dose of NTG represents a “supraphysiological” stimulus, it can nonetheless be effectively reversed by standard doses of a proven migraine-specific therapy. Similarly, the ability of topiramate to inhibit the chronic basal hyperalgesia induced by chronic NTG indicates that this model may also be used to test potential migraine prophylactics.

NTG evoked acute and chronic basal hyperalgesia is a promising mouse model for the investigation of migraine mechanisms and therapies. The enhancement of acute NTG-evoked hyperalgesia by a human migraine-associated gene expressed in mice, and the

inhibition of this hyperalgesia by both acute and preventive migraine therapies provide strong support for the translational value of this model. Ongoing studies of the effects of other human migraine genes, as well as other established acute and preventive therapies, will help to further validate the model as a useful tool for enhancing our ability to understand and treat this frequently disabling disorder.

Conflict of interest statement

The authors report no conflict of interest.

Acknowledgments

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RESEARCH PAPER

δ -Opioid receptor agonists inhibit migraine-related hyperalgesia, aversive state and cortical spreading depression in mice

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BACKGROUND AND PURPOSE

Migraine is an extraordinarily common brain disorder for which treatment options continue to be limited. Agonists that activate the δ -opioid receptor may be promising for the treatment of migraine as they are highly effective for the treatment of chronic rather than acute pain, do not induce hyperalgesia, have low abuse potential and have anxiolytic and antidepressant properties. The aim of this study was to investigate the therapeutic potential of δ -opioid receptor agonists for migraine by characterizing their effects in mouse migraine models.

EXPERIMENTAL APPROACH

Mechanical hypersensitivity was assessed in mice treated with acute and chronic doses of nitroglycerin (NTG), a known human migraine trigger. Conditioned place aversion to NTG was also measured as a model of migraine-associated negative affect. In addition, we assessed evoked cortical spreading depression (CSD), an established model of migraine aura, in a thinned skull preparation.

KEY RESULTS

NTG evoked acute and chronic mechanical and thermal hyperalgesia in mice, as well as conditioned place aversion. Three different δ -opioid receptor agonists, SNC80, ARM390 and JNJ20788560, significantly reduced NTG-evoked hyperalgesia. SNC80 also abolished NTG-induced conditioned place aversion, suggesting that δ -opioid receptor activation may also alleviate the negative emotional state associated with migraine. We also found that SNC80 significantly attenuated CSD, a model that is considered predictive of migraine preventive therapies.

CONCLUSIONS AND IMPLICATIONS

These data show that δ -opioid receptor agonists modulate multiple basic mechanisms associated with migraine, indicating that δ -opioid receptors are a promising therapeutic target for this disorder.

Abbreviations

ARM390, N,N-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide; CSD, cortical spreading depression; JNJ20788560, 9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9H-xanthene-3-carboxylic acid diethylamide; NTG, nitroglycerin; SNC80, (+)-4-[(α R)- α -(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethyl benzamide

Introduction

Migraine is a highly prevalent neurological disorder that affects over 800 million people worldwide (Stovner *et al.*, 2007; Bigal *et al.*, 2008; Stewart *et al.*, 2008; Victor *et al.*, 2010). The basic mechanisms underlying migraine remain inadequately understood, and while available therapies are effective for some, there are a large number of patients for whom current treatments are ineffective or poorly tolerated (Stovner *et al.*, 2007; Bigal *et al.*, 2008).

Most opioid analgesics in common clinical use for migraine and other pain conditions primarily target the μ -opioid receptor (receptor nomenclature follows Alexander *et al.*, 2013). However, these μ -receptor agonists have relatively poor efficacy as analgesics for migraine headache, and can contribute to progression of migraine from an episodic phenomenon to a frequent or daily condition that is refractory to other therapies (Bigal and Lipton, 2008; Bigal *et al.*, 2008). This 'medication overuse headache' is a particularly difficult clinical problem and is a common pathway to abuse of prescription μ -opioid receptor agonists.

The δ -opioid receptor agonists have markedly different properties from those of the μ -agonists. While compounds that selectively activate δ -opioid receptors have been found to be less effective as analgesics in acute pain models, they have shown efficacy in assays of chronic inflammatory (Fraser *et al.*, 2000; Cahill *et al.*, 2003; Petrillo *et al.*, 2003; Pradhan *et al.*, 2009; 2010) and neuropathic (Petrillo *et al.*, 2003; Kabli and Cahill, 2007; Gaveriaux-Ruff *et al.*, 2011) pain. In addition, δ -agonists do not induce the same types of adverse events as μ -agonists, such as respiratory depression and constipation (Negus *et al.*, 1994; Gallant and Meert, 2005). Further, we have shown that unlike pharmacotherapies targeting the μ -opioid receptor, hyperalgesia does not occur following chronic administration with δ -agonists (Pradhan *et al.*, 2010). As an additional advantage, activation of the δ -opioid receptor does not appear to be highly rewarding, thus decreasing the abuse potential of δ -opioid receptor agonists. Finally δ -opioid receptors positively modulate emotional state. Genetic deletion of either the δ -opioid receptor or its endogenous ligand, enkephalin, results in anxiogenic and depressive-like behaviours (Konig *et al.*, 1996; Filliol *et al.*, 2000). Conversely, δ -agonists produce anxiolytic and antidepressant effects (Saitoh *et al.*, 2004; Perrine *et al.*, 2006). This emotional modulation may be particularly important for the treatment of migraine pain, as there is a high co-morbidity of migraine with depression and anxiety (Silberstein *et al.*, 2007). Although their characteristics seem well suited for treatment of migraine, δ -agonists have not yet been systematically investigated as a therapy for this disorder.

The aim of this study was to investigate the potential of δ -opioid receptor agonists as anti-migraine therapies. We found that δ -opioid receptor agonists given to mice modulate several basic mechanisms associated with migraine, indicating that these receptors are a promising therapeutic target for this disorder.

Methods

Animals

All animal care and experimental procedures were approved by the University Of California Los Angeles Office of Animal Research, in accordance with AALAC guidelines. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to *in vivo* techniques, if available. A total of 225 animals were used in the experiments described here.

We used male and female C57BL6/J mice (Jackson Laboratories, Bar Harbor, ME, USA), and δ -opioid receptor knockout mice (backcrossed onto a C57BL6 background; Brigitte Kieffer, IGMBC, Illkirch, France) weighing 20–30 g. Animals were housed in a 12 h light–dark cycle, and food was available *ad libitum*. For experiments using δ -opioid receptor knockout mice, heterozygous pairs were bred to obtain wild-type and knockout animals (Filliol *et al.*, 2000).

Drug administration

Nitroglycerin (NTG) was prepared from a stock solution of 5.0 mg·mL⁻¹ NTG in 30% alcohol, 30% propylene glycol and water (American Regent, Shirely, NY, USA). NTG was freshly diluted in 0.9% saline to a dose of 10 mg·kg⁻¹. The vehicle used in these experiments was 0.9% saline. We found that there was no significant difference in mechanical thresholds between 0.9% saline and the solution in which NTG was dissolved (6% propylene glycol, 6% alcohol, 0.9% saline). All injections were administered as a 10 mL·kg⁻¹ volume. Unless otherwise noted, animals were tested for baseline responses immediately before *i.p.* injection with NTG. Animals were injected *i.p.* with vehicle, SNC80 ((+)-4-[(α R)- α -(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethyl benzamide) or sumatriptan, or *per os* with vehicle, ARM390 (N,N-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide) or JNJ20788560 (9-(8-azabicyclo[3.2.1]oct-3-ylidene)-9H-xanthene-3-carboxylic acid diethylamide), 1 h 15 min–1 h 30 min following NTG injection and were tested for mechanical or thermal sensitivity 30–45 min later (2 h post-NTG).

Sensory sensitivity testing

To determine mechanical sensitivity, the threshold for responses to punctate mechanical stimuli (mechanical hyperalgesia) was tested according to the up-and-down method (Chaplan *et al.*, 1994). In brief, the plantar surface of the animal hindpaw was stimulated with a series of eight von Frey filaments (bending force ranging from 0.008 to 2 g). A response was defined as a lifting or shaking of the paw upon stimulation. The first filament tested was 0.4 g. In the absence of a response, a heavier filament (up) was tried, and in the presence of a response, a lighter filament (down) was tested. This pattern was followed for a maximum of four filaments following the first response. To assess thermal responses, heat sensitivity was assessed by immersing the tail (5 cm from the tip) into a 46°C water bath. Tail withdrawal latencies were determined, and a cut-off of 40 s was established. For all pain experiments, groups were counterbalanced based on their naïve baselines (basal responses taken on day 1).

Conditioned place aversion

Independent groups of mice were trained for NTG-induced place conditioning and tested either treatment-free or under treatment effects (state dependency). We used a two-chamber conditioned place paradigm, which is described in detail by Tzschentke (2007). This consists of two identical Plexiglas shuttle boxes divided into two distinct compartments. The shuttle boxes consist of two large compartments separated by a plastic guillotine door. The compartments differ in floor texture (metal grated floors with a horizontal or vertical pattern) and wall pattern (horizontal or vertical black and white stripes). Live video tracking was used to collect data on time spent in each chamber and analysed using Noldus Ethovision software (Leesburg, VA, USA).

NTG conditioning consisted of three phases. For the pre-conditioning phase, on day 1, naïve mice were allowed to freely explore the apparatus for 15 min. Based on the results of this preconditioning, the drug-paired chambers were assigned in a counterbalanced and unbiased manner to saline and NTG groups. During the conditioning phase, on days 2, 4, 6 and 8, animals were injected with saline and restricted to one of the conditioning chambers 1 h 50 min to 2 h 10 min post-injection. On days 3, 5, 7 and 9, mice were injected with saline (VEH group) or 10 mg·kg⁻¹ i.p. NTG (NTG group), and were restricted to the other conditioning chamber 1 h 50 min to 2 h 10 min post-injection. For experiments where animals received a second treatment of vehicle (0.9% saline) or SNC80 (10 mg·kg⁻¹ SNC80 i.p.), these compounds were administered 35 min prior to the conditioning session. For the post-conditioning test phase, on day 10, animals were allowed free access to both chambers in a drug/hyperalgesia-free state and the time spent in each chamber was recorded (PAIN/DRUG-FREE). On day 11, animals were given their assigned drug treatments and given free access to the entire chamber as a test of state-dependent aversion (STATE-DEPENDENT).

Cortical spreading depression

Mice were randomly assigned to treatment or control groups. Each animal was transferred to the surgical area and weighed prior to the start of the experiment. Gas mixture of 2:1 nitrogen to oxygen was used throughout the duration of the experiment. Anaesthesia was induced with 5% isoflurane, which was immediately lowered to ~2% for the placement into the stereotaxic frame and the surgery. During recordings of cortical spreading depression (CSD), isoflurane was maintained at ~1.5%. To insure anaesthesia level was stable, we waited at least 1 h between the surgery and the start of the experiment. Anaesthesia level was assessed throughout the experiment by monitoring breathing rate (80 to 110 times per minute) and response to tail pinch. Temperature was established with the rectal probe and maintained at 37 ± 0.7°C.

After induction of anaesthesia, the skin from the skull was detached and a rectangular region of ~2.5 × 3.3 mm² (~0.5 mm from sagittal, and ~1.4 from coronal and lamboid sutures) of the right parietal bone was thinned to transparency with a micro drill (Fine Science Tools, Inc., Foster City, CA, USA). To improve transparency, a thin layer of silicon oil was applied over the skull. The brain was illuminated with a green LED (540 nm). Images were captured with a resolution of 1040 × 1392 at 1 Hz with a High-Sensitivity USB Monochrome CCD Camera (Mightex Systems, Pleasanton, CA,

USA) through a 4× objective (UPlanSApo; Olympus, Center Valley, PA, USA).

The burr hole was placed outside of and lateral to the midpoint of the thinned rectangular region. A MicroFil syringe needle with 1 M KCl was placed into the burr hole, avoiding contact with brain or bone. The initial drop of KCl stimulation was formed by using a pneumatic pico-pump with the pressure at ~20 psi. Afterward, even flow of KCl was obtained using constant holding pressure of ~0.8 psi, to maintain a pool of 1 M KCl for the duration of the experiment. This resulted in a CSD frequency of 10–14 CSDs per hour in control animals. Excess liquid was removed with tissue paper applied next to the burr hole. The first CSD was elicited within 80 s of the recording. At ~400 s, mice were injected with either saline or saline plus SNC80 (10.0 mg·kg⁻¹, administered 1 mg·mL⁻¹ i.p.), and the recording was continued for an additional 3600 s, resulting in a total recording duration of 4000 s. Experiments were included in analysis only if at least two CSD events occurred during the first 400 s (prior to administration of either saline or SNC80).

Data analysis

Data are expressed as mean ± SEM. All statistical analyses were performed by Sigmapstat software (Systat Software Inc., San Jose, CA, USA). For all acute pain experiments, one-way ANOVA was performed. For mutant mouse experiments, a two-way ANOVA was used, and for chronic pain experiments, a two-way repeated measures ANOVA was performed. For conditioned place aversion, CPA magnitude was defined as the difference between time spent on the treatment-paired and vehicle-paired sides on the test days, and a *t*-test was performed for the NTG-induced CPA experiment, while a two-way ANOVA was performed to determine the effect of δ -agonists on NTG-induced CPA. Unless otherwise noted, all experiments were further analysed using Holm–Sidak *post hoc* analysis.

Materials

The drugs used in these experiments were supplied as follows; ARM390 provided by AstraZeneca R&D (Montreal, Canada); isoflurane by Piramal Critical Care Inc (Bethlehem, PA); JNJ20788560 provided by Johnson & Johnson (Spring House, PA); SNC80 by Tocris (Bristol, UK); sumatriptan by Zogenix (San Diego, CA)

Results

Mechanical and thermal hyperalgesia induced by acute NTG is reversed by selective δ -agonists

NTG is a known migraine trigger in humans, and acute systemic administration of NTG has been shown to produce mechanical and heat hyperalgesia in mice that is inhibited by the anti-migraine drug sumatriptan (Bates *et al.*, 2010; Pradhan *et al.*, 2013). We first determined whether selective δ -agonists could reverse acute NTG-evoked hyperalgesia. Two selective δ -agonists were tested: SNC80 and ARM390. Both compounds show similar receptor binding and G protein activation properties, and produce comparable anti-hyperalgesic effects in models of inflammatory pain.

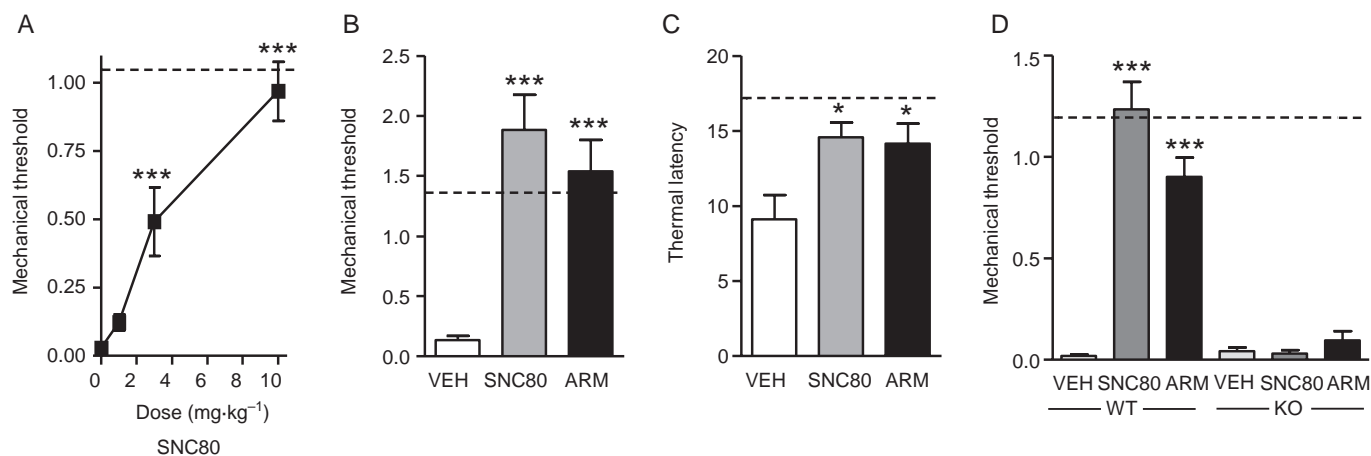


Figure 1

Acute treatment with NTG produced mechanical and thermal hyperalgesia, which was reversed by δ -opioid receptor agonists. In all cases, before NTG (10 mg·kg⁻¹, i.p.) administration, baseline mechanical or thermal responses were determined (dashed lines and below mean \pm SEM). δ -Agonists were administered 1 h 15 min after NTG injection, and animals were tested 45 min later (2 h post-NTG). (A) The selective δ -agonist, SNC80, dose dependently reversed NTG-induced mechanical hyperalgesia. $n = 8$ –10 per group, *** $P < 0.001$, significantly different from vehicle controls, one-way ANOVA. Naïve baseline (1.30 \pm 0.12). At maximal doses, both SNC80 (10 mg·kg⁻¹), and another δ -agonist, ARM390 (10 mg·kg⁻¹), produced comparable reversals of NTG-induced mechanical (B, naïve baseline 1.36 \pm 0.09) and thermal hyperalgesia (C, naïve baseline 17.16 \pm 0.59) $n = 6$ per group, * $P < 0.05$, *** $P < 0.001$; significantly different from vehicle controls, one-way ANOVA. (D) Neither SNC80 nor ARM390 (10 mg·kg⁻¹) were effective in δ -opioid receptor knockout mice. $n = 4$ –5 per group, *** $P < 0.001$, two-way ANOVA. Naïve baseline (1.2 \pm 0.13). For mechanical responses, the Y axis indicates the 50% mechanical threshold to respond.

However, the two drugs produce distinct forms of analgesic tolerance, correlated with distinct receptor internalizing properties (see Pradhan *et al.*, 2009).

NTG (10 mg·kg⁻¹, i.p.) produced marked mechanical hypersensitivity in mice compared with baseline responses (Figure 1A, vehicle – 0 mg·kg⁻¹ vs. dashed line), and SNC80 dose dependently reversed this hyperalgesia (Figure 1A). In a separate group of animals, a maximal doses of ARM390 (10 mg·kg⁻¹) also reversed NTG-induced mechanical hyperalgesia, comparable to the effect of SNC80 (10 mg·kg⁻¹, Figure 1B). In addition, systemic administration of NTG also induced heat hyperalgesia (Figure 1C, VEH vs. dashed line), which was also reversed by both SNC80 and ARM390 (Figure 1C). We further examined the selectivity of both δ -agonists in the NTG model, by testing δ -opioid receptor wild-type and knockout mice. We found that the effects of SNC80 and ARM390 in reversing NTG-induced mechanical hyperalgesia in wild-type mice were absent in the δ -receptor knockout animals (Figure 1D). In addition, the δ -opioid receptor antagonist, naltrindole, blocked the anti-hyperalgesic effects of SNC80 and ARM390 in wild-type mice (Supporting Information Fig. S1). Thus, selective δ -opioid receptor agonists reverse sensory hypersensitivity in the NTG model of migraine pain.

δ -Opioid receptor agonists alleviate chronic NTG-evoked hyperalgesia

As with other types of pain, migraine can progress from an episodic to a chronic condition. We therefore examined the effectiveness of repeated administration of δ -opioid receptor agonists in the treatment of hyperalgesia evoked by chronic

intermittent administration of NTG. To model the progression to chronic migraine, we administered NTG (10 mg·kg⁻¹, i.p.) every second day for 9 days, resulting in a total of five NTG injections/test days. Each injection of NTG produced marked mechanical hyperalgesia, as observed in the vehicle treated groups (Figure 2A and B). Both SNC80 and ARM390 inhibited the hyperalgesia that followed each dose of NTG (Figure 2A). There appeared to be tolerance to SNC80 and ARM390 following day 1; however, this day appeared to be unusually high, and there was no significant difference of the effect of these compounds days 2–5. We also tested the novel δ -opioid receptor agonist, JNJ20788560 at a dose that was previously shown to inhibit CFA-induced hyperalgesia (Codd *et al.*, 2009). As with the other two δ -agonists, JNJ20788560 significantly inhibited the NTG-evoked hyperalgesia (Figure 2B). Similar results were observed in response to treatment with sumatriptan, a prototypic anti-migraine medication (Figure 2C). These results indicate that δ -agonists maintain efficacy in reversing the effects of NTG, even with repeated administration.

δ -Agonists alleviate the aversive experience induced by NTG

We tested the ability of SNC80 and ARM390 to alleviate the aversive experience produced by NTG by using a conditioned place aversion paradigm. The concept underlying these experiments is that if pain/discomfort is consistently paired with a certain location, that location ultimately becomes aversive.

We first determined whether NTG produced a conditioned place aversion. Animals were tested in a two-chamber

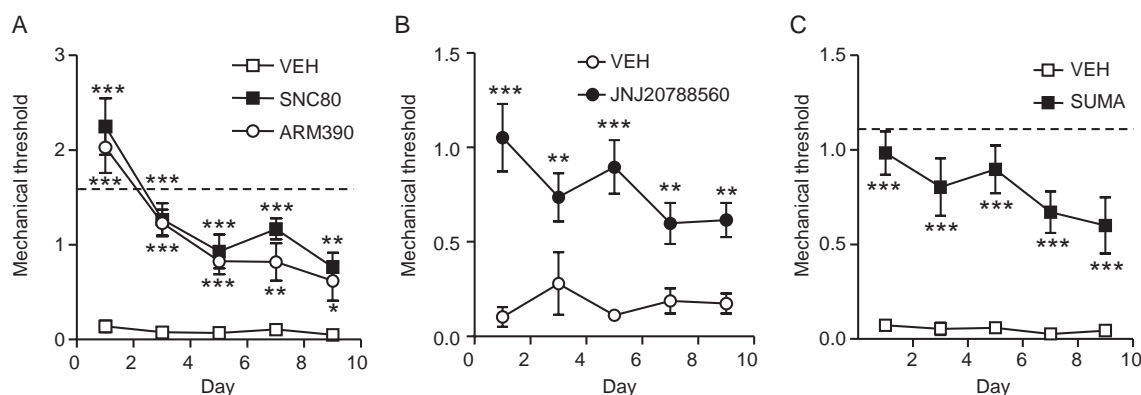


Figure 2

Chronic NTG-evoked hyperalgesia is attenuated by δ -opioid receptor agonists. C57Bl/6J mice were treated every second day with NTG ($10 \text{ mg} \cdot \text{kg}^{-1}$, i.p.) for 9 days. NTG injection produced mechanical hyperalgesia (VEH), which was significantly attenuated by SNC80 and ARM390 (A, $10 \text{ mg} \cdot \text{kg}^{-1}$), JNJ20788560 (B, $30 \text{ mg} \cdot \text{kg}^{-1}$) and the anti-migraine drug, sumatriptan (C, $600 \mu\text{g} \cdot \text{kg}^{-1}$). $n = 5\text{--}11$ per group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-way repeated measures ANOVA. Dashed lines represent mean basal responses as determined on day 1 (1.5 ± 0.10 , 1.17 ± 0.08 and 1.11 ± 0.14 for panels A, B and C respectively). The Y axis indicates the 50% mechanical threshold to respond.

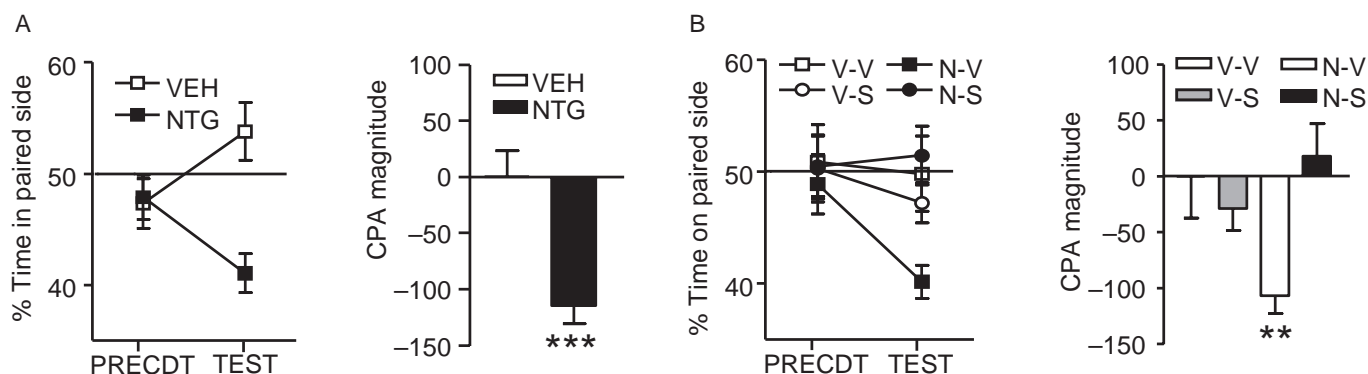


Figure 3

Chronic NTG produced conditioned place aversion, which was reversed by treatment with SNC80. Mice were tested following the conditioning phase in a state-dependent test. (A) NTG treated animals showed a clear (left panel) and significant (right panel) conditioned place aversion $n = 8$ per group, $***P < 0.001$, Student's t -test. CPA magnitude was defined as the difference between time spent on the NTG-paired and VEH-paired sides on the test days. (B) Animals were conditioned with vehicle-vehicle (V-V), vehicle-SNC80 ($10 \text{ mg} \cdot \text{kg}^{-1}$ i.p., V-S), NTG ($10 \text{ mg} \cdot \text{kg}^{-1}$, i.p.)-vehicle (N-V) or NTG-SNC80 ($10 \text{ mg} \cdot \text{kg}^{-1}$, N-S). In a state-dependent test, NTG produced a significant conditioned place aversion, which was reversed by SNC80 (right panel). $n = 8\text{--}9$ per group, $**P < 0.01$, two-way ANOVA. CPA magnitude was defined as the difference between time spent on the treatment-paired and VEH-VEH-paired sides on the test days.

conditioning paradigm. On the preconditioning day, no significant preference for either chamber was observed, and mice were assigned to either NTG ($10 \text{ mg} \cdot \text{kg}^{-1}$ i.p.) or vehicle treatment groups (Figure 3A left panel, PRECDT). Following the conditioning sessions, animals were tested in treatment/drug-free state, and neither an aversion nor preference was observed following chronic NTG or vehicle treatment (Supporting Information Fig. S2A). We then determined if an aversion could be observed during a state-dependent test. In this case, animals were tested following treatment with either NTG or vehicle, and given free access to both compartments. NTG-conditioned animals showed a significant aversion to the treatment-paired compartment during this state-dependent test (Figure 3A, right panel). Thus, NTG produces

a state-dependent aversion within the conditioned place aversion paradigm.

We next tested if a δ -agonist could reverse NTG-induced aversion. Again, no initial preference was observed during the preconditioning test (Figure 3B left panel, PRECDT). During the conditioning period, animals were injected twice: first with NTG or vehicle and 1.15 h later with SNC80 ($10 \text{ mg} \cdot \text{kg}^{-1}$ i.p.) or vehicle. During the treatment-free test, neither aversion nor preference was observed in any of the treatment groups (Supporting Information Fig. S2B). However, during the state-dependent test, NTG animals subsequently treated with vehicle showed a significant conditioned place aversion, which was not observed in mice treated with NTG followed by SNC80 (Figure 3B, right panel).

CSD is inhibited by a δ -opioid receptor agonist

To investigate the effects of δ -opioid receptor agonists on mechanisms of cortical excitability underlying migraine, we quantified the effects of SNC80 on repetitive CSD evoked by continuous application of KCl, a model that has previously been shown to have predictive value for migraine preventive medications (Ayata *et al.*, 2006; Bogdanov *et al.*, 2011). CSD events were visualized as waves of optical intrinsic signals emanating from the site of KCl application. In previous studies, these were shown to be invariably associated with electrophysiological changes consistent with CSD (Brennan *et al.*, 2007a,b; Chang *et al.*, 2010). Either saline or SNC80 were injected 400 s after the initiation of KCl application, and recordings were continued for an additional 3600 s. Neither SNC80 nor saline resulted in any significant change in respiratory rate or pulse rate (consistent with previous studies).

The number of CSD events before and after administration of SNC80 was counted based on kymographs of optical intrinsic signal and verified by evaluation of original video recordings. The number of CSD events occurring in the 1 h time interval following injection of SNC80 was significantly less than the number of events that occurred in the same time period following saline (Figure 4). We did not observe any significant differences in the amplitude, duration, or propagation rate of CSD events following SNC80 versus saline injection (not shown). We also investigated if acute or chronic NTG treatment altered the frequency of repetitive CSDs evoked by continuous KCl stimulation. Previous studies indicated that acute NTG did not alter the threshold for KCl-evoked stimulation (Bates *et al.*, 2010). Consistent with this result, we did not observe any significant differences in the frequency of CSD events in mice treated acutely with NTG or chronically with NTG using the protocol described above (Supporting Information Fig. S3).

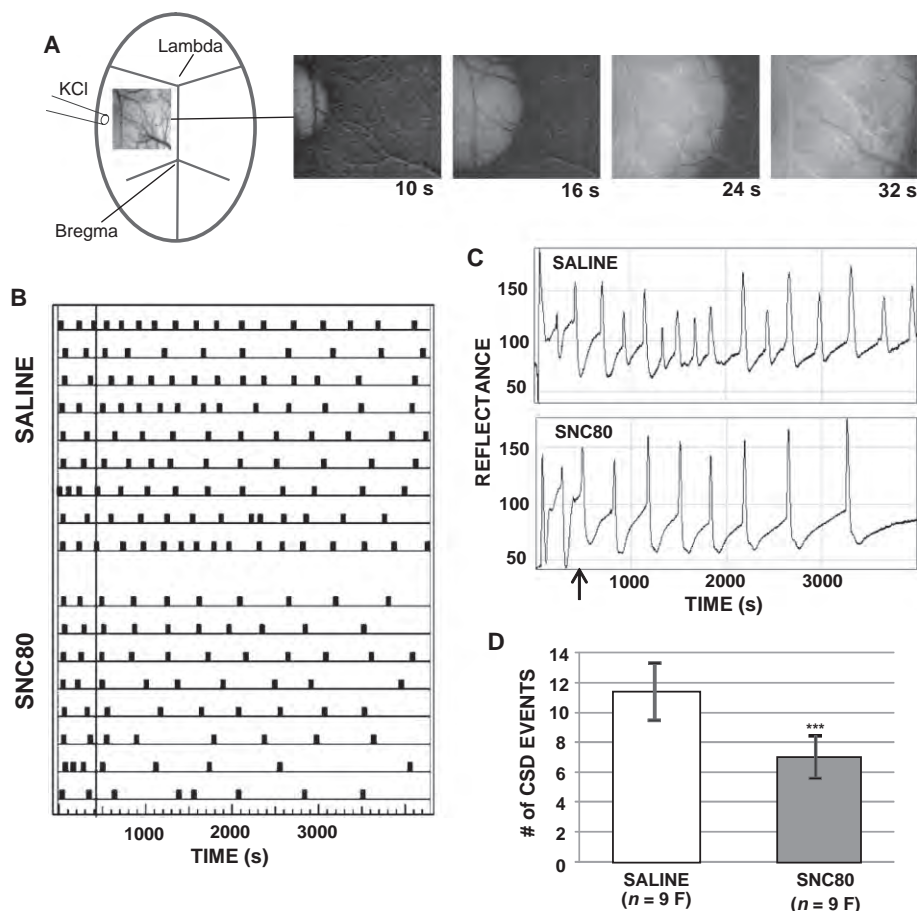


Figure 4

SNC80 inhibits CSD. (A) The schematic drawing on the left indicates the window of thinned skull through which CSD was imaged and the location of KCl injection. The image sequence on the right shows the wave of change in reflectance associated with a typical CSD event. (B) Data from all imaging experiments showing the occurrence of CSD events over time. Each horizontal line represents the results from an individual mouse, with CSD events indicated by the vertical bars. Either saline or SNC80 were injected i.p. at 400 s (indicated by vertical line), and the recording was then continued for an additional 10 min. (C) Line tracings of reflectance versus time for typical individual experiments shown in B, with injection of either saline or SNC80 at 400 s (indicated by arrow). Following injection of SNC80, there is a reduced number of CSD events as compared with following injection of saline. (D) Graph shows the average number of CSD events over 10 min following injection of saline or SNC80. Treatment with SNC80 resulted in a significant reduction in the number of CSD events ($P < 0.001$, Student's *t*-test).

Discussion

Our results show that δ -opioid receptor agonists are promising potential therapies for migraine. In an acute and chronic model of migraine pain, SNC80 and ARM390 effectively reversed hyperalgesia evoked by the known migraine trigger NTG. In addition, SNC80 prevented NTG-induced conditioned place aversion suggesting that δ -agonists may also have the potential to treat the negative emotional state induced by migraine. Furthermore, in a well-established model of migraine aura, SNC80 caused a decrease in the number of CSD events, a characteristic shared with migraine prophylactics. Together, these results make a strong case for the development of δ -agonists for the treatment of migraine.

Previous characterization of δ -agonists show that they are effective in many other models of chronic pain (Fraser *et al.*, 2000; Cahill *et al.*, 2003; Petrillo *et al.*, 2003; Kabli and Cahill, 2007; Pradhan *et al.*, 2009; 2010), and do not produce opioid-induced hyperalgesia (Pradhan *et al.*, 2010). Furthermore, δ -opioid receptor activation positively modulates emotional tone, and δ -agonists are being tested clinically for the treatment of anxiety and depression. Considering the high level of co-morbidity between migraine and emotional disorders (Merikangas *et al.*, 1993; Breslau *et al.*, 1994), δ -agonists may be well positioned as novel migraine therapies.

NTG is a reliable migraine trigger in susceptible patients (Christiansen *et al.*, 1999; Afridi *et al.*, 2005), and NTG-evoked sensory hypersensitivity in rodents has been developed as a model for the cutaneous allodynia (Bates *et al.*, 2010; Pradhan *et al.*, 2013), photophobia and vasodilation of meningeal blood vessels (Greco *et al.*, 2011; Markovics *et al.*, 2012) associated with migraine. In addition, in a transgenic mouse model of familial migraine, mice expressing a human migraine gene showed an even greater sensitivity to NTG-evoked hyperalgesia (Brennan *et al.*, 2013). Furthermore, the prototypic anti-migraine drug, sumatriptan, was shown to inhibit acute NTG-induced hyperalgesia (Bates *et al.*, 2010; Pradhan *et al.*, 2013), and we further validated its effects in our chronic NTG model. Similar to sumatriptan, three distinct δ -agonists SNC80, ARM390, and JNJ20788560, all inhibited hyperalgesia evoked by acute and chronic intermittent NTG administration. There is the possibility that the anti-hyperalgesic effects of δ -agonists may be mediated only through the spinal cord and dorsal root ganglia. However, δ -opioid receptors are expressed in numerous supraspinal regions within the pain matrix, including the trigeminal nucleus caudalis, the amygdala and the cortex (Mansour *et al.*, 1988; Pradhan and Clarke, 2005; Pradhan *et al.*, 2011), indicating that these receptors can regulate pain processing at a number of different levels. Importantly, the ability of SNC80 to reduce NTG-induced conditioned place aversion and CSD events suggest that δ -agonists are acting on multiple anatomical sites regulating migraine.

In this study, we tested agonists with differing abilities to induce δ -opioid receptor internalization (Pradhan *et al.*, 2009). Both the high-internalizing agonist (SNC80) and the low internalizing agonist (ARM390) equally reduced hyperalgesia in a peripheral inflammatory pain model (Pradhan *et al.*, 2009; 2010), and our results show that this is also the case in a model of migraine pain. These results would suggest that the anti-hyperalgesic effects of δ -agonists are due to

G-protein-dependent signalling, and not to signalling cascades activated following receptor sequestration. We have also previously shown that repeated treatment with δ -agonists results in tolerance (Pradhan *et al.*, 2010), which would limit their use for chronic disorders. However, in those experiments, SNC80 and ARM390 were administered daily for 5 days. In this manuscript, animals were treated every second day, and tolerance was attenuated. Although we observed a drop in effectiveness following day 1, responses on this day appeared to be unusually high, and there was no significant decrease in drug effect on days 2–5. This differing response could reflect experimenter or environmental factors particular to that day, or habituation to the test apparatus following the first treatment day. Further, we did not observe tolerance to the δ -agonist JNJ20788560. Overall, it appears that giving the animals a 48 h drug-free period between injections may be sufficient to prevent adaptations that result in tolerance to δ -agonists. In addition, tolerance was not observed to δ -opioid receptor agonists following chronic treatment within other behavioural paradigms (Brandt *et al.*, 2001; Petrillo *et al.*, 2003; Beaudry *et al.*, 2009; Codd *et al.*, 2009; Pradhan *et al.*, 2010), indicating that both the drug, the drug regimen and the disease model are important aspects to consider in the development of δ -agonists for chronic use.

We have also extended the characterization of the nociceptive effects of NTG administration by demonstrating that it conditions an aversive association from which an animal learns to avoid associated contextual cues. Measurement of the response evoked by heat or mechanical stimulation is a commonly used pain model, but this type of measurement does not consistently correlate with analgesic efficacy (Vierck *et al.*, 2008; King *et al.*, 2009) nor does it capture the emotional dysfunction that is characteristic of chronic pain states (Merikangas *et al.*, 1993; Breslau *et al.*, 1994). The conditioned place aversion assay is designed to address these issues (Hummel *et al.*, 2008). We found that mice showed significant state-dependent place aversion following administration of NTG. State-dependent learning refers to the facilitation of a preference or aversion response during the time in which the animal is in the same state that has been used to produce the learned response (see (Tzschentke, 2007)). The δ -agonist, SNC80, abolished NTG-evoked place aversion. SNC80 alone has been previously reported to produce conditioned place preference (Longoni *et al.*, 1998); or under other circumstances, conditioned taste aversion (Hutchinson *et al.*, 2000). In our study, however, SNC80 (vehicle-SNC80 group, Figure 3) produced neither preference nor aversion, most likely due to differences in experimental design. There is also the possibility that the locomotor stimulant effect of SNC80 obscured the expression of conditioned place aversion to NTG. However, previous work has shown that the locomotor stimulants, morphine and cocaine, both showed enhanced conditioned place preference in state-dependent tests (Bespalov *et al.*, 1999; Dockstader and van der Kooy, 2001; Harris *et al.*, 2001). Together with our results, this data suggests that hyperlocomotion does not override state-dependent learning and retrieval.

Multiple migraine preventive medications with diverse pharmacological mechanisms have been shown to inhibit CSD (Ayata *et al.*, 2006). Other studies indicate that the CSD model may only be predictive of medications effective in

migraine with aura, rather than migraine in general (Bogdanov *et al.*, 2011). The effects of SNC80 on CSD could be the result of direct action in the cortex, on sub-cortical modulators of cortical excitability, or both. δ -Opioid receptors are widely expressed in the cortex (Mansour *et al.*, 1988; Pradhan and Clarke, 2005; Pradhan *et al.*, 2011), but their cortical function is unclear. Enkephalins, endogenous agonists at the δ -opioid receptor, have been reported to have both excitatory and inhibitory effects on cortical excitability and CSD in rodents (Sprick *et al.*, 1981; Oitzl *et al.*, 1985). Dual effects of δ -agonists on cortical excitability could be explained by direct inhibition of the cortex versus an indirect excitation mediated by inhibition of either cortical or subcortical inhibitory neurons. Regardless of the underlying mechanism, the inhibition of CSD by SNC80 in our studies suggests that δ -agonists may have migraine preventive properties, in addition to the acute therapeutic properties indicated by the NTG models.

CSD and NTG-induced behaviours likely represent distinct migraine mechanisms. In this study, and in another (Bates *et al.*, 2010), NTG did not alter CSD measurements. CSD is a well-established model of migraine aura, a condition experienced only by a subset of migraineurs. NTG administration to patients with migraine, even those who have migraine with aura, does not typically induce aura. It is therefore not necessarily surprising that NTG does not alter CSD propensity, and we suggest that NTG activates migraine pain pathways through mechanisms that are distinct from changes in cortical excitability that cause migraine aura. Migraine is a multidimensional phenomenon that affects multiple brain and peripheral regions (Charles, 2013).

μ -Opioid receptor agonists such as codeine, hydrocodone, oxycodone, meperidine and morphine, are commonly used in North America as 'rescue' therapy for migraine (Bigal and Lipton, 2009). In Europe and elsewhere in the world, the use of μ -agonists for migraine has been significantly curtailed because of the recognition that these medications not only have relatively poor efficacy as acute migraine therapies, but that they also have significant abuse potential and can play a role in progression of migraine to a chronic state (Evers *et al.*, 2009). In contrast to μ -agonists, δ -agonists do not produce adverse effects such as respiratory suppression, sedation or reduction in gastric motility (Gallantine and Meert, 2005; see Pradhan *et al.*, 2011). To date, there are no human studies examining the abuse liability of δ -agonists. In non-human primates, however, δ -agonists appear to have low abuse liability as SNC80 is not self-administered (Negus *et al.*, 1998; Stevenson *et al.*, 2005) nor does it cause dependence (Brandt *et al.*, 2001). In addition, in a rodent model of abuse liability, SNC80 did not alter intracranial self-stimulation responses (Do Carmo *et al.*, 2009). δ -Agonists are therefore an appealing alternative to the μ -agonists currently being used to treat migraine.

There is a significant need for new acute and preventive treatments for migraine, and there is an additional need for better translational models to identify novel treatments. Given the complexity and heterogeneity of migraine, it is unlikely that any single model will adequately predict efficacy of therapies. Our studies using several behavioural and physiological models identify the δ -opioid receptor as a promising therapeutic target for migraine. Several δ -opioid

receptor compounds are currently in development for clinical use and our results indicate that clinical trials of these compounds are warranted for this highly prevalent and frequently disabling condition.

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Conflicts of interest

The authors declare no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 The anti-hyperalgesic effects of SNC80 and ARM390 were blocked by the δ -antagonist, naltrindole. NTG (10 mg·kg⁻¹ i.p.) was injected immediately after baseline mechanical responses were determined (dashed lines, 1.07 ± 0.11), and 15 min later mice were injected with vehicle (V) or naltrindole (N, 10 mg·kg⁻¹ s.c.). SNC80 (S) or ARM390 (A) (10 mg·kg⁻¹) was administered 1 h 15 min post-NTG and mice were injected 45 min later. $n = 4–6$ mice per group, * $P < 0.05$, ** $P < 0.01$, t -test with Bonferroni correction. SNC80 and ARM390 are selective for the δ -opioid receptor.

Figure S2 Chronic NTG did not produce a significant condition place aversion in a treatment-free test. Mice were tested following the conditioning phase treatment-free. (A) In a treatment-free test, conditioning with vehicle or NTG (10 mg·kg⁻¹, i.p.) did not produce a significant change in % time spent on the paired side (left panel) or conditioned place aversion (CPA) magnitude (right panel). CPA magnitude was defined as the difference between time spent on the NTG-paired and VEH-paired sides on the test days. (B) In a treatment-free test, conditioning with vehiclevehicle (V-V), vehicle-SNC80 (10 mg·kg⁻¹ i.p., V-S), NTG (10 mg·kg⁻¹, i.p.)-vehicle (N-V) or NTGSNC80 (10 mg·kg⁻¹, N-S) did not produce a significant change in % time spent on the paired side (left panel) or CPA magnitude (right panel). CPA magnitude was defined as the difference between time spent on the treatment-paired and VEH-VEH-paired sides on the test days.

Figure S3 Graph shows the average number of CSD events over 10 min in saline-treated mice versus mice treated with chronic nitroglycerin (10 mg·kg⁻¹ i.p.) over 9 days (as in hyperalgesia assays) versus acute nitroglycerin (10 mg kg⁻¹ i.p.) during an imaging experiment. There was no significant difference between the number of CSD events under these conditions.